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(54) Title: METHOD FOR THE PRODUCTION OF NON-IMMUNOGENIC PROTEINS

(57) Abstract

Protein, or parts of proteins, may be rendered non-immunogenic, or less immunogenic, to a given species by identifying in their amino acid sequences one or more potential epitopes for T-cells of the given species and modifying the amino acid sequence to eliminate at least one of the T-cell epitopes. This eliminates or reduces the immunogenicity of the protein when exposed to the immune system of the given species. Monoclonal antibodies and other immunoglobulin-like molecules can particularly benefit from being de-immunised in this way: for example, mouse-derived immunoglobulins can be de-immunised for human therapeutic use.

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METHOD FOR THE PRODUCTION OF NON-IMMUNOGENIC PROTEINS

The present invention relates to the production of substantially non-immunogenic proteins, especially antibodies, and their uses. The invention uses a combination of recombinant DNA and monoclonal antibody technology for the generation of novel therapeutic and *in vivo* diagnostic agents for particular use in man.

The use of rodent, especially mouse, monoclonal antibodies for therapeutic and *in vivo* diagnostic applications in man was found to be limited by immune responses made by patients to the rodent antibody. The development of so-called "HAMA" (human anti-mouse antibody) responses in patients was shown to limit the ability of antibodies to reach their antigenic targets resulting in a reduced effectiveness of the antibodies. In order to reduce the HAMA response, chimaeric antibodies were developed (see, for example, WO-A-8909622) in which the mouse variable (V) regions were joined to human constant (C) regions. Such antibodies have proved clinically useful although the mouse V region component still provides the basis for generating immunogenicity in patients (see, for example, LoBuglio *et al.*, *Proc. Nat'l. Acad. Sci. USA* 86 4220-4224 (1989)). Therefore, technology for humanised antibodies were developed whereby the complementarity determining regions or "CDRs" from the rodent antibody were transplanted onto human V regions and joined to human C regions to create humanised antibodies whereby the only non-human components were the CDRs which were adjacent to human V region "frameworks". The transplanted CDRs corresponded either to hypervariable regions as defined by Kabat *et al.* ("Sequences of Proteins of Immunological Interest", Kabat E., *et al.* , US Dept. of Health and Human Services, 1983) or to the hypervariable loops in 3-dimensional structures of antibodies (Chothia and Lesk, *J. Mol. Biol.* 196 901-917 (1987)). One of the first examples of such humanised antibodies by Riechmann *et al.* (*Nature* 332 323-326 (1988)) illustrated, however, that simple transplantation of CDRs often resulted in reduced affinity of the humanised antibody and consequently that the introduction of certain

non-human amino acids (i.e. from the corresponding position in the rodent sequence) in the human V region framework as required in order to restore affinity. A number of methods have been proposed for the substitution of human framework residues in order to restore affinity including those disclosed in EP-A-0239400, EP-A-0438310, WO-A-9109967 and WO-A-9007861. In particular, patent publications by Protein Design Labs., Inc. (*e.g.* WO-A-9007861 and related EP-B-0451216) purport to provide a general method for producing humanised antibodies in which one or more human framework residues are altered in order to restore binding affinity.

A common aspect of all of the above mentioned methods for production of chimeric or humanised antibodies is that the objective of these methods was to create antibodies which are substantially non-immunogenic in humans (*e.g.* EP-B-0451216, p3, line 6). However, the means for achieving this objective has been the introduction into the rodent antibody of as much human sequence as possible and it has been assumed that such a general introduction of human sequence will render the antibodies non-immunogenic. It is known that certain short peptide sequences ("epitopes") can be immunogenic in humans and none of the methods for chimaeric or humanised antibodies have considered how to eliminate or avoid such epitopes in the resultant antibody. Furthermore, most of the methods (*e.g.* EP-B-0451216) have advocated the introduction of non-human amino acids into human V region frameworks without considering the possible creation of immunogenic epitopes, and none of the methods has provided any means for avoiding or eliminating immunogenic epitopes at framework:CDR junctions and, where practical, within CDRs themselves. Thus, of the methods devised with the objective of creating substantially non-immunogenic antibodies, none can be considered as actually achieving the creation of such substantially non-immunogenic antibodies. The same can be said of proteins (especially therapeutic proteins) other than antibodies.

The present invention provides, for the first time, a general method for creating substantially non-immunogenic proteins such as antibodies and also provides antibodies and other proteins created by this method.

According to a first aspect of the invention, there is provided a method of rendering a protein, or part of a protein, non-immunogenic, or less immunogenic, to a given species, the method comprising:

(a) determining at least part of the amino acid sequence of the protein;

(b) identifying in the amino acid sequence one or more potential epitopes for T-cells ("T-cell epitopes") of the given species; and

(c) modifying the amino acid sequence to eliminate at least one of the T-cell epitopes identified in step (b) thereby to eliminate or reduce the immunogenicity of the protein or part thereof when exposed to the immune system of the given species.

The term "T-cell epitopes" refers to specific peptide sequences which either bind with reasonable efficiency to MHC class II molecules or which, from previous or other studies, show the ability to stimulate T-cells *via* presentation on MHC class II. However, it will be understood that not all such peptide sequences will be delivered into the correct MHC class II cellular compartment for MHC class II binding or will be suitably released from a larger cellular protein for subsequent MHC class II binding. It will also be understood that even such peptides which are presented by MHC class II on the surface of antigen-presenting cells will elicit a T cell response for reasons including a lack of the appropriate T cell specificity and tolerance by the immune system to the particular peptide sequence.

Potential epitopes for B-cells of the given species may additionally be compromised in a similar manner.

The invention has particular application to rendering regions of immunoglobulins non-immunogenic (which term will be used in this specification to include less immunogenic, unless the context dictates otherwise): constant or, especially, variable regions of immunoglobulins (or of course natural or artificial molecules containing both such regions) constitute proteins, or parts of proteins, to which the invention is well suited to being applied.

However, it will be understood to those skilled in the art that the present invention could also be applied to produce therapeutic proteins other than immunoglobulins or antibodies. As with antibodies, proteins which would otherwise be immunogenic in man could be de-immunised by removal of T cell epitopes. In addition, if a reference human protein is available with similar secondary structure and identifiable surface amino acids, the B cell epitopes could additionally be removed from the protein by substituting surface amino from the reference human protein in place of the corresponding amino acids in the non-human or potentially immunogenic protein. For example, clinical use of the thrombolytic agent bacterial streptokinase is limited by human immune responses against the molecule; such molecules could be engineered to remove potential T cell epitopes in order to remove the immunogenicity.

Generally, the invention will be used to reduce the immunogenicity of a protein or part thereof (exemplified by a V region of an immunoglobulin) of a first species in relation to the immune system of a second species. The first species may be non-human, and the second species may be human. Examples of typical non-human species useful in relation to embodiments of the invention relating to immunoglobulins include mammals, especially rodents such as rats and, in particular, mice, and farm animals such as sheep and cattle. However, as made clear above in relation to bacterial streptokinase, the first species may be taxonomically far removed from the second

species; when the first species is non-human, it may be non-mammalian and even non-eukaryotic. In much of the following description of preferred embodiments of the invention, reference will be made to humanising antibodies, but it is to be understood that the invention also relates to species other than man and to proteins, particularly therapeutic proteins, generally, including specific binding molecules other than whole antibodies.

The method of the invention is based on the consideration of how an immune response against a monoclonal is usually created in humans as the basis for avoiding or eliminating sequences within the antibody which are involved in this immune response. When a therapeutic antibody or other immunoglobulin, or partial immunoglobulin, molecule ("antibody", for short) is administered to a human patient, the antibody is subjected to surveillance by both the humoral and cellular arms of the immune system which will respond to the antibody if it is recognised as foreign and if the immune system is not already tolerant to the immunogenic sequence within the antibody. For the humoral immune response, immature B-cells displaying surface immunoglobulins (sIg) can bind to one or more sequences within the therapeutic antibody ("B-cell epitopes") if there is an affinity fit between the an individual sIg and the B-cell epitope and if the B-cell epitope is exposed such that sIg can access the B-cell epitope. The process of sIg binding to the therapeutic antibody can, in the presence of suitable cytokines, stimulate the B-cell to differentiate and divide to provide soluble forms of the original sIg which can complex with the therapeutic antibody to limit its effectiveness and facilitate its clearance from the patient. However, for an effective B-cell response, a parallel T-cell response is required in order to provide the cytokines and other signals necessary to give rise to soluble antibodies. An effective T-cell response requires the uptake of the therapeutic antibody by antigen-presenting cells (APCs) which can include B-cells themselves or other professional APCs such as macrophages, dendritic cells and other monocytes. In addition, non-professional APCs such as the cells to which the antibody binds can take up the therapeutic antibody and provide intermediate processing of the antibody such

that professional APCs can then absorb the antibody components. Having taken up the therapeutic antibody, APCs can then present suitable peptides from the therapeutic antibody ("T-cell epitopes") complexed with MHC class II molecules at the cell surface. Such peptide-MHC class II complexes can be recognised by helper T-cells via the T-cell receptor and this results in stimulation of the T-cells and secretion of cytokines which provides "help" for B-cells in their differentiation to full antibody producing cells. In addition, the T-cell response can also result in deleterious effects on the patient for example through inflammation and allergic reactions.

An effective primary immunogenic response to a therapeutic antibody therefore usually requires a combination of B- and T-cell responses to B- and T-cell epitopes. Therefore, avoidance of a primary immunogenic response requires the avoidance or elimination of both B- and T-cell epitopes within the therapeutic antibody. Without either the B- or T-cell response, the primary immunogenic response to a therapeutic antibody is likely to be muted or absent. The present invention therefore provides methods for avoiding or eliminating T-cell epitopes, or a combination of both B- and T-cell epitopes, from therapeutic antibodies in order to create substantially non-immunogenic antibodies with particular emphasis on avoiding such epitopes in the V region of the therapeutic antibody. For B-cell epitopes, the method takes advantage of the fact sIg can only bind to accessible regions of the therapeutic antibody, *i.e.* sequences of exposed surface amino acids. For a starting mouse antibody for subsequent human use, the method then incorporates into the V region of the therapeutic antibody, human amino acids at positions corresponding to those of the exposed mouse amino acids. For T-cell epitopes, sequences of overlapping peptides within the therapeutic antibody are analysed, with particular emphasis on the V region, in order to identify putative peptides suitable for presentation by MHC class II molecules. By scanning the V region of a potential therapeutic antibody and, where T-cell epitopes are identified, changing one or more individual amino acids to eliminate the T-cell epitope, then an antibody can be created devoid of T-cell epitopes. For the C regions of the therapeutic antibody or other immunoglobulin molecule, contiguous

natural C regions from human antibodies can be used, although the invention also encompasses the identification and elimination of T-cell epitopes in the C regions if desirable or necessary.

It will be understood that the invention is not just applicable to whole antibodies, but rather to any specific binding molecule comprising a V region of an immunoglobulin, including without limitation whole Ig light (κ and λ) and heavy (γ, α, μ, δ and ε) chains, light/heavy chain dimers, SCAs (single-chain antibodies), and antibody or immunoglobulin fragments including those designated Fab, $F(ab')_2$, Fab', Fd and Fv.

While the usefulness of the invention is not confined to making an antibody or other V region-containing molecule of one particular species therapeutically or diagnostically administrable to any other particular species, the most significant utility of the invention will be in "humanising" non-human antibodies, particularly rodent antibodies such as murine antibodies (or parts of them). In that case, the "first species" referred to above will be a mouse, and the "second species" will be a human.

Therefore, a particular embodiment of the present invention comprises the following key steps:

(a) determining the amino acid sequence of the V region of a starting antibody, which will usually be non-human, *e.g.* mouse;

(b) optionally modifying the amino acid sequence, for example by recombinant DNA techniques, to change those non-CDR residues on the exposed surface of the antibody structure to the corresponding human amino acids taken from a reference (*e.g.* closely matched) human V region sequence (which may be a human germ-line V region sequence);

(c) analysing the amino acid sequence to identify potential T-cell epitopes and modifying the amino acid sequence, for example by recombinant DNA techniques, to change one or more residues in order to eliminate at least some, and preferably all, of the T-cell epitopes, particularly framework epitopes but including those within CDRs if this does not undesirably reduce or eliminate binding affinity or undesirably alter specificity; and

(d) optionally adding human C regions via recombinant DNA to create a complete antibody which is substantially non-immunogenic.

A preferred method of the present invention therefore combines the removal of both B- and T-cell epitopes from a therapeutic antibody, a process which is termed "de-immunisation". For removal of human B-cell epitopes from the V region of a therapeutic antibody, the method of Padlan (Padlan E.A., *Molecular Immunology* 28 489-498 (1991) and EP-A-0519596) provides a suitable procedure whereby surface amino acids in a particular antibody sequence are identified with reference to 3-dimensional structures or models of antibody V regions and are converted to the corresponding human residues in a process which has been called "veneering". A derivative of this method (EP-A-0592106) models the V regions of the therapeutic antibody itself in order to identify surface amino acids in a process which has been called "resurfacing".

The present invention provides for removal of human (or other second species) T-cell epitopes from the V regions of the therapeutic antibody (or other molecule) whereby the sequences of the V region can be analysed for the presence of MHC class II-binding motifs by any suitable means. For example, a comparison may be made with databases of MHC-binding motifs such as, for example by searching the "motifs" database at the world-wide web site *wehil.wehi.edu.au*. Alternatively, MHC class II-binding peptides may be identified using computational threading methods such as

those devised by Altuvia *et al.* (*J. Mol. Biol.* **249** 244-250 (1995)) whereby consecutive overlapping peptides from the V region sequences are testing for their binding energies to MHC class II proteins. In order to assist the identification of MHC class II-binding peptides, associated sequence features which relate to successfully presented peptides such as amphipathicity and Rothbard motifs, and cleavage sites for cathepsin B and other processing enzymes can be searched for.

Having identified potential second species (*e.g.* human) T-cell epitopes, these epitopes are then eliminated by alteration of one or more amino acids, as required to eliminate the T-cell epitope. Usually, this will involve alteration of one or more amino acids within the T-cell epitope itself. This could involve altering an amino acid adjacent the epitope in terms of the primary structure of the protein or one which is not adjacent in the primary structure but is adjacent in the secondary structure of the molecule. The usual alteration contemplated will be amino acid substitution, but it is possible that in certain circumstances amino acid addition or deletion will be appropriate. All alterations can for preference be accomplished by recombinant DNA technology, so that the final molecule may be prepared by expression from a recombinant host, for example by well established methods, but the use of protein chemistry or any other means of molecular alteration is not ruled out in the practice of the invention.

In practice, it has been recognised that potential human T-cell epitopes can be identified even in human germ-line V region framework sequences when comparison is made with databases of MHC-binding motifs. As humans do not generally mount an ongoing immune response against their own antibodies, then either humans are tolerant to these epitopes or these potential epitopes cannot be presented by human APCs because they are not processed appropriately. Therefore, such potential T-cell epitopes which are represented in germ-line V region sequences may, in practice, be retained in the de-immunised antibody. In order to minimise the creation of additional T-cell epitopes during the elimination of potential T-cell epitopes from the therapeutic antibody sequence, the elimination of T-cell epitopes is preferably (but not

necessarily) achieved by conversion to second species (usually human) germ-line amino acids at positions corresponding to those of the first species (usually mouse) amino acids within T-cell epitopes. Once initially identified T-cell epitopes are removed, the de-immunised sequence may be analysed again to ensure that new T-cell epitopes have not been created and, if they have, the epitope(s) can be deleted, as described above; or the previous conversion to a corresponding human germ-line amino acid is altered by conversion of the murine (or other first species) amino acid to a similar non-human (or non-second species) amino acid (*i.e.* having similar size and/or charge, for example) until all T-cell epitopes are eliminated.

For the C region of a therapeutic de-immunised antibody or other molecule subjected to the method of the invention, it is not necessary to systematically eliminate potential B- and T-cell epitopes as the use of contiguous natural human C region domains has so far proved safe and substantially non-immunogenic in patients; thus the combination of de-immunised V regions and human C regions is sufficient for creation of a substantially non-immunogenic antibody or other immunoglobulin V region-containing molecule. However, as human C regions have sites of amino acid allotypic variation which might create potential T-cell epitopes for some allotypes, then the method of Lynxvale Ltd. (Clark) published in WO-A-9216562 and EP-A-0575407 might be useful. Equally, the method of the invention may be applied to a C region in a similar manner as it is applied to a V region.

For the CDRs of a therapeutic antibody, it is common for one or more potential T-cell epitopes to overlap or fall within the CDRs whereby removal of the epitopes requires alteration of residues within the CDRs. In order to eliminate the induction of a T-cell response to such epitopes, it is desirable to eliminate these although this may reduce the binding affinity of the resultant antibody and thus any potential alteration of CDRs may need to be tested for any alteration of resultant antigen binding.

A typical therapeutic de-immunised antibody from the present invention will comprise heavy and light chain V region sequences (V_H, V_L) with several amino acid substitutions which constitute departures from the prototype rodent sequence. Typically, for a V_H or V_L region, there will be 10 to 15 substitutions with human residues to eliminate B-cell epitopes and 1 to 10 human or non-human substitutions to eliminate T-cell epitopes. The typical therapeutic de-immunised antibody will also comprise human C regions for the heavy and light chains.

EP-B-0451216 discloses

> the use of at least one amino acid substitution outside of complementarity determining regions (CDRs) as defined...in the production of a humanized immunoglobulin, wherein said amino acid substitution is from the non-CDR variable region of a non-human donor immunoglobulin, and in which humanized immunoglobulin the variable region amino acid sequence other than the CDRs comprises at least 70 amino acid residues identical to an acceptor human immunoglobulin variable region amino acid sequence, and the CDRs are from the variable region of said non-human donor immunoglobulin.

In certain preferred de-immunised antibodies of the present invention, the variable region amino acid sequence other than the CDRs comprises fewer than 70 amino acid residues identical to an acceptor human immunoglobulin variable region amino acid sequence (*i.e.* a reference human variable region sequence such as a germ-line variable region sequence).

EP-B-0451216 also discloses

> a method of producing a humanized immunoglobulin chain having a framework region from a human acceptor immunoglobulin and complementarity determining regions (CDR's) from a donor immunoglobulin

capable of binding to an antigen, said method comprising substituting at least one non-CDR framework amino acid of the acceptor immunoglobulin with a corresponding amino acid from the donor immunoglobulin at a position in the immunoglobulins where:

(a) the amino acid in the human framework region of the acceptor immunoglobulin is rare for said position and the corresponding amino acid of the donor immunoglobulin is common for said position in human immunoglobulin sequences; or

(b) the amino acid is immediately adjacent to one of the CDR's; or

(c) the amino acid is predicted to have a side chain capable of interacting with the antigen or with the CDR's of the humanized immunoglobulin.

In the present invention, preferred de-immunised antibody variable region amino acid sequence other than CDRs would exclude amino acids from the starting antibody which are rare at the corresponding position in human immunoglobulins or which are adjacent to CDRs or which have a side-chain capable of interacting with the antigen or with the CDRs of the de-immunised antibody.

It will be understood by those skilled in the art that there can be several variations of the method of the present invention which will fall within the scope of the present invention. Whilst the present invention relates principally to therapeutic antibodies from which human B- and T-cell epitopes have been deleted, it will be recognised that the removal of T-cell epitopes alone might, in some cases, also be effective in avoiding an immunogenic response in patients. As an alternative to the de-immunised antibodies of the present invention, part of the method of the first aspect of the present invention may be used to analyse pre-existing antibodies in therapeutic use in order to predict the basis for immunogenic responses to these antibodies and to eliminate them by induction of B- or T-cell tolerance to the appropriate B- and T-cell epitopes or by

other methods for ablating the immune response. In addition, it should be considered within the scope of the present invention to redesign a pre-existing therapeutic antibody to which a human immune response has been detected and characterised to delete the epitopes relating to the observed immune response in humans. Additionally, as discussed above, therapeutic and other proteins apart from antibodies may benefit from the application of the invention.

It should be understood that the method of the present invention could be used to render a V region of an immunoglobulin either wholly non-immunogenic or partially immunogenic, whereby certain B- or T-cell epitopes may be left within the final molecule in order to elicit an immune reaction in patients, for example with an anti-idiotype antibody where only usually part of the V region is involved in mimicking the original antigen. It should also be understood that the present invention can apply to the production of antibodies for uses other than in human medicine and that de-immunised antibodies could be produced for specific therapeutic or diagnostic use in animals whereby de-immunisation eliminates the specific animal's B- and T-cell epitopes.

As indicated above, the method of the present invention may also be used to render constant regions of immunoglobulins non-immunogenic. For example, in a typical humanisation of a non-human antibody, instead of incorporating a human constant region into the final molecule, the non-human constant region could be screened for the presence of T cell epitopes which would then be eliminated preferably without altering any of the biochemical properties of the constant region such as the ability to fix complement. Alternatively, the equivalent human biological properties could be deliberately incorporated into the de-immunised constant regions by incorporating corresponding human residues, for example for binding to efficient binding to human Fc receptors. If required, certain properties of non-human constant regions could be retained in the de-immunised constant regions, for example to retain the co-operative binding effect of mouse IgG3 antibodies.

According to a second aspect of the invention, there is provided a molecule of a first species (such as a non-human species), wherein the variable region is modified to eliminate epitopes for T-cells, and optionally also epitopes for B-cells, of a second species (such as human). The molecule will generally be proteinaceous and may comprising at least a variable region of an immunoglobulin, in which case the first species may be mouse. The variable region may be modified to the minimum extent necessary to eliminate the T-cell epitopes. Alternatively or additionally, it may be modified to eliminate only T-cell epitopes which are non-germ-line.

The invention extends also to a molecule which has been prepared by a method in accordance with the first aspect of the invention.

The invention has particular and widespread application in the field of therapeutic molecules including monoclonal antibodies whereby rodent or other non-human antibodies can be de-immunised for applications in humans and whereby previously humanised or chimaeric antibodies with B- or T-cell epitopes could be converted into a less immunogenic form for use in humans. It will also be understood that even antibodies derived from human immunoglobulin genes such as antibodies derived from bacteriophage-display libraries (Marks *et al.*, *J. Mol. Biol.* **222** 581-597 (1991)), transgenic mice with human immunoglobulin genes (Bruggermann *et al.*, *Proc. Nat'l. Acad. Sci. USA* **86** 6709-6713 (1989)) and natural human monoclonal antibodies can carry B- and T-cell epitopes especially as somatic mutations are introduced into framework sequences in immunoglobulin genes during the maturation of antibodies. Therefore, de-immunisation may be required in order to prepare such antibodies for use in humans. Finally, it will be understood that CDRs from any naturally derived antibodies have been subjected to selection by somatic mutation of V region genes and thus might have T-cell epitopes capable of triggering immune responses in humans. The de-immunisation method might be applicable without severe loss of antibody binding affinity (depending on the contribution of particular CDRs to antigen binding).

According to a third aspect of the invention, there is provided a molecule which has been prepared by a method in accordance with the first aspect of the invention, or a molecule in accordance with the second aspect, for use in medicine or diagnosis.

According to a fourth aspect of the invention, there is provided the use of a molecule prepared by a method in accordance with the first aspect of the invention, or a molecule in accordance with the second aspect, in the manufacture of an therapeutic or diagnostic antibody or other specific binding molecule. The invention therefore extends to a method of treating or preventing a disease or condition, the method comprising administering to a subject an effective amount of a molecule prepared by a method in accordance with the first aspect of the invention, or a molecule in accordance with the second aspect. The invention also extends to the use of such molecules in *in vivo* and *in vitro* diagnosis.

Preferred features of each aspect of the invention are as for each other aspect, *mutatis mutandis*.

The invention will now be illustrated, but not limited, by the following examples. The examples refer to the drawings, in which:

FIGURE 1 shows the DNA sequences of 340 V_H and V_L;

FIGURE 2 shows the protein sequence of 340 murine V_H and V_L;

FIGURE 3 shows the protein sequence of humanised 340 V_H and V_L;

FIGURE 4 shows oligonucleotides for construction of humanised 340 V_H and V_K;

FIGURE 5 shows the protein sequence of de-immunised 340 V_H and V_L;

FIGURE 6 shows oligonucleotides for construction of de-immunised 340 V_H and V_K;

FIGURE 7 shows the comparative binding of humanised, de-immunised and chimaeric antibody to an epidermal growth factor receptor (EGFR) preparation from;

FIGURE 8 shows the protein sequence of humanised 340 V_H compared with the sequence with murine epitopes inserted;

FIGURE 9 shows oligonucleotide primers for insertion of murine epitopes into humanised 340 V_H by SOE PCR;

FIGURE 10 shows the protein sequence of mouse de-immunised 340 V_H;

FIGURE 11 shows oligonucleotide primers for construction of mouse de-immunised V_H;

FIGURE 12 shows the primary and secondary immunogenic responses to antibodies in accordance with the invention and contrasts them with immunogenic responses to antibodies not within the scope of the invention;

FIGURE 13 shows DNA sequences of murine 708 V_H and V_L;

FIGURE 14 shows protein sequences of murine 708 V_H and V_L;

FIGURE 15 shows DNA sequences of de-immunised 708 V_H and V_L;

FIGURE 16 shows oligonucleotides for construction of de-immunised 708 V_H and V_L;

FIGURE 17 shows protein sequences of Vaccine 1 708 V_H and V_L;

FIGURE 18 shows oligonucleotides for construction of Vaccine 1 708 V_H and V_L;

FIGURE 19 shows protein sequences of Vaccine 2 708 V_H and V_L;

FIGURE 20 shows oligonucleotides for construction of Vaccine 2 708 V_H and V_L;

FIGURE 21 shows protein sequences of Vaccine 3 708 V_H;

FIGURE 22 shows oligonucleotides for construction of Vaccine 3 708 V_H;

FIGURE 23 shows oligonucleotides for construction of chimaeric 708 V_H and V_L;

FIGURE 24 shows the protein sequence of humanised A33 V_H and V_L;

FIGURE 25 shows the protein sequence of de-immunised humanised A33 V_H and V_L;

FIGURE 26 shows the protein sequence of murine A33 VH and VL;

FIGURE 27 shows the protein sequence of de-immunised murine A33 V_H and V_L;

FIGURE 28 shows the protein sequence of streptokinase from *Streptococcus equisimilis*; and

FIGURE 29 shows the protein sequence of a de-immunised streptokinase molecule.

Example 1

mRNA was isolated from $5x10^6$ hybridoma 340 cells (Durrant *et al.*, *Prenatal Diagnostics*, 14 131 (1994) using TRIzol™ reagent (Life Technologies, Paisley, UK) according to the manufacturer's instructions. The mRNA was converted to cDNA/mRNA hybrid using Ready-To-Go™ T-primed First-Strand kit (Pharmacia Biotech, St. Albans, UK). Variable region heavy (V_H) and light (V_L) chain cDNAs were amplified using primer sets using the method of Jones and Bendig (*Bio/Technology*, 9 188 (1991). PCR products were cloned into pCRII (Invitrogen, Netherlands) and six individual clones each of V_H and V_L were sequenced in both directions using the Applied Biosystems automated sequencer model 373A (Applied Biosystems, Warrington, UK). Resultant V_H and V_L DNA sequences are shown in Figure 1 and the corresponding protein sequences in Figure 2.

A humanised antibody was generated by substituting the mouse V region frameworks 1 to 3 for corresponding frameworks from the human germ-line V region sequences HSIGDP54 (Tomlinson *et al.*, *J. Mol. Biol.*, 227 776 (1992) for V_H and HSIGKV38 (Victor *et al.*, *J. Clin. Invest.*, 87 1603 (1991)) for V_L. For the 4th framework, the human J_H6 was substituted in the V_H and the human J_k4 in the VL. In addition, some key amino acids from the murine sequences which were expected to be important to restore binding in the humanised antibody were substituted for the corresponding human framework residues. The amino acid sequences of the humanised V_H and V_L are shown in Figure 3.

The humanised V_H and V_L regions were constructed by the method of overlapping PCR recombination using long synthetic oligos described by Daugherty *et al.*, (*Nucleic Acids Research*, 19 2471 (1991)). The required sequence was synthesised as

four long oligonucleotides of 96 to 105bp with complementary overlapping ends of 18 base pairs (Figure 4). These were used in PCR with two external primers resulting in the formation and subsequent amplification of full length V regions (363 bp for V_H and 330 bp for V_K). DNAs of the vectors M13-VHPCR1 and M13-VKPCR1 (Orlandi *et al., Proc. Nat'l. Acad. Sci. USA*, **86** (1989)) were used as templates to produce a further two overlapping PCR fragments for each of V_H and V_L including 5' flanking sequence with the murine heavy chain immunoglobulin promoter and encoding the leader signal peptide and 3' flanking sequence including a splice site and intron sequences. The DNA fragments so produced for each of V_H and V_L were combined in a second PCR using outer flanking primers to obtain the required full length DNA sequences.

The humanised VH gene complete with 5' and 3' flanking sequences was cloned into the expression vector, pSVgpt (Riechmann *et al., Nature*, **332** 323 (1988)) which includes the human IgG1 constant region domain (Takahashi *et al., Cell*, **29** 671 (1982)) and the gpt gene for selection in mammalian cells. The humanised V_L gene complete with 5' and 3' flanking sequences was cloned into the expression vector, pSVhyg (Riechmann *et al., ibid.*), in which the gpt gene is replaced by the gene for hygromycin resistance (hyg) and a human kappa constant region is included (Hieter *et al., Cell*, **22** 197 (1980)).

The heavy and light chain expression vectors were co-transfected into NS0, a non-immunoglobulin producing mouse myeloma, obtained from the European Collection of Animal Cell Cultures, Porton Down, UK, ECACC No 85110505, by electroporation. Colonies expressing the gpt gene were selected in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) FCS and antibiotics (Life Technologies Ltd, Paisley, UK) and with 0.8 μg/ml mycophenolic acid and 250 μg/ml xanthine (Sigma, Poole, UK).

Production of human antibody by transfected cell clones was measured by ELISA for human IgG (Tempest *et al., Bio/Technology*, 9 266 (1991)). Cell lines secreting antibody were expanded and antibody purified by protein A affinity chromatography (Harlow E, Lane D; in "Antibodies, a Laboratory Manual", Cold Spring Harbor Laboratory (1988) page 309).

A de-immunised antibody was generated by analysis of the sequence of Figure 2. To remove B cell epitopes, the "veneering" method of Padlan (Padlan E.A. , *Molecular Immunology* **28** 489-498 (1991) and EP-A-0519596) was applied whereby exposed (mE or Ex) residues in the murine 340 V_H and V_L sequences were substituted by the corresponding residues in the frameworks from the human germ-line V region sequences HSIGDP54 for V_H and HSIGKV38 for V_L. Then, the resultant sequences were analysed by searching a database of human MHC class II binding peptides ("motif" at the world-wide web site *wehil.wehi.edu.au*) for motifs present in the veneered V_H and V_L sequences. In parallel, databases of human V_H and V_L germ-line sequences (Tomlinson *et al.*, *ibid.*; Cox *et al.*, *Eur. J. Immunol.*, **24** 827 (1994); other germ-line sequences retrieved from EMBL, GenBank and Swiss Protein databases) were also searched for human MHC class II binding motifs. Motifs appearing in the veneered antibody sequence which were also present in the germ-line were not considered further. For motifs present in the veneered V_H and V_L sequences and not present in the germ-line database, single amino acid substitutions to the corresponding human germ-line sequences were made in order to delete the motif unless a substitution was required within a CDR. Following this round of motif deletion, the resultant sequence was checked for generation of new MHC class II binding motifs and these were similarly deleted if present. The resultant de-immunised V_H and V_L sequences are shown in Figure 5. The de-immunised V_H and V_L regions were constructed as above by the method of Daugherty *et al.* (*ibid.*) using oligonucleotides synthesised with adjacent 18 nucleotide overlaps as detailed in Figure 6. Cloning, sequencing, addition of C regions and expression in NS0 cells was as for the humanised antibody.

A chimaeric antibody comprising murine 340 V_H and V_L regions and human IgG1/kappa C regions was generated as detailed in Orlandi *et al.*, *ibid*.

Comparative antibody binding to an epidermal growth factor receptor (EGFR) preparation from placenta. 30-40g of human placenta was washed in PBS containing phenyl methyl sulphonyl, chopped finely, homogenised, lysed in 1% NP-40 and centrifuged at 10,000g for 10 minutes. The supernatant was then loaded onto a CNBr-activated antibody 340 column (2mg antibody per ml of gel) and eluted fractions were monitored by SDS-PAGE and protein analysis. ELISA plates were coated with fractions of EGFR preparation to give OD450 of 1.0 with murine 340 antibody using anti-mouse IgG peroxidase conjugate (Sigma). 1005µl serial dilutions of the test recombinant antibodies and an irrelevant humanised antibody were incubated overnight in the ELISA plates and detected using peroxidase-labelled gamma chain-specific anti-human IgG antibody (Sigma). Results are shown in Figure 7 and these show that the de-immunised antibody bound to the EGFR antigen with similar efficiency to the chimaeric antibody with the humanised antibody displaying an approximate five-fold deficit in binding.

Example 2

In this example, a range of antibodies were tested in mice to compare immune responses. As a source of antibody to elicit an immune response in mice, the humanised V_H fragment from Example 1 was deliberately altered to insert two murine MHC class II epitopes as shown in Figure 8. This was undertaken by SOE PCR (Higuchi *et al.*, *Nucleic Acids Research*, 16 7351 (1988)) using primers as detailed in Figure 9. Using methods as in Example 1, for the murine de-immunised version the MHC class II epitopes were removed from the altered humanised V_H fragment and this was also veneered to substitute exposed residues from the murine 340 sequence. The resultant sequence is shown in Figure 10 and the synthetic oligonucleotides used shown in Figure 11.

The murine de-immunised V_H fragment from above and the humanised and murine V_H fragments from Example 1 were joined either to human or murine C region fragments of isotype IgG2. For human, a 7.2kb *Hin*dIII-*Bam*HI genomic fragment from IgG2 C region (Bruggemann *et al.*, *J. Exp. Med.*, 166 1351 (1987)) was used and, for murine, a 4.2kb *Eco*RI-*Bgl*II fragment from mouse IgG2b^b (Ollo and Rougeon, *Nature*, 296 761 (1982)) was used. Fragments were blunt-ended using the Klenow fragment of DNA polymerase and *Bgl*II linkers were added (according to the manufacturer's instructions (New England Biolabs, Beverly, Mass, USA) for cloning into the *Bam*HI site of pSVgpt (Riechmann, *ibid.*). Recombinant plasmids were transfected by electroporation into J558L cells which secrete lambda light chains. Antibodies were purified from culture supernatants by protein A affinity chromatography as above.

To study immune responses, groups of five 6-8 week-old female BALB/c or C57BL/6 mice were injected intraperitoneally with 40μg of recombinant antibody or murine 340 antibody in CFA. Serum was taken for analysis after 30 days and mice were boosted with the same antibodies in IFA; serum was again taken 10 days later. Antibody responses were measured in ELISA assays with the immobilised antibody used for immunisation. Dilutions of sera were added and incubated for 2hrs at 37c. Binding was then detected using biotinylated anti-mouse kappa chain antibody (Harlan-Seralab, Crawley, UK) and HRP-streptavidin (Pierce and Warriner, Chester, UK) according to the supplier's instructions. Colour was developed with OPD (o-phenylenediamine) substrate (Sigma, Poole, UK). The results were expressed as serial dilutions from an average of 5 mice per group (SD<20%) which gave half maximum binding to immobilised antibody on the ELISA plate.

The results are shown in Figure 12 which shows a strong primary and secondary immunogenic response to the antibodies with the humanised but not the De-immunised or murine V_H regions and a murine heavy chain C region (lanes 1, 2 and 3 respectively). For the de-immunised V_H with a human heavy chain C region (lane 4),

a considerable primary and secondary immune response was found which was absent with the mouse 340 antibody control (lane 5).

Example 3

mRNA was isolated from 5×10^6 hybridoma 708 cells (Durrant *et al.*, *Int. J. Cancer*, 50 811 (1992) using TRIZOL™ reagent (Life Technologies, Paisley, UK) according to the manufacturers' instructions. The mRNA was converted to cDNA/mRNA hybrid using READY-TO-GO™ T-primed First Strand Kit (Pharmacia Biotech, St. Albans, UK). Variable region heavy (V_H) and light (V_L) chain cDNAs were amplified using the primer sets using the method of Jones and Bendig (*Bio/Technology*, 9 188 (1991)). PCR products were cloned into pBLUESCRIPT II SK⁻ (Stratagene, Cambridge, UK) or pCRTM 3 (Invitrogen, The Netherlands) and six individual clones each of V_H and V_L were sequenced ion both directions using the Applied Biosystems automated sequencer model 373A (Applied Biosystems, Warrington, UK). Resultant V_H and V_L sequences are shown in Figure 13 and the corresponding protein sequences in Figure 14.

A de-immunised antibody was generated by analysis of the sequence of Figure 14. To remove B cell epitopes, the "veneering" method of Padlan (Padlan E.A., *Molecular Immunology* **28** 489 (1991) and EP-A-0519596) was applied whereby exposed (mE or Ex) residues in the murine 708 V_H or V_L sequences were substituted by the corresponding residues in the frameworks from the human germ-line sequences DP-30 for V_H (Tomlinson *et al.*, *J. Mol. Biol.* **227** 776 (1992) with human J_H1 and DPK-1 (Cox *et al.*, *Eur. J. Immunol.*, **24** 827 (1994)) for V_L with human J_K4. Then, the resultant sequences were analysed by searching a database of human MHC Class II binding peptides ("motif" at the World Wide Web site *wehil.wehi.edu.ac*) for motifs present in the veneered V_H and V_L sequences. In parallel, databases of human V_H and V_L germ-line sequences (Tomlinson *et al.*, *ibid.*; Cox *et al. ibid.*; other germ-line sequences retrieved from EMBL, GenBank and Swiss Protein databases) were also

searched for human MHC Class II binding motifs. Motifs appearing in the veneered antibody sequence which were also present in the germ-line were not considered further. For motifs present in the veneered V_H and V_L sequences and not present in the germ-line database, single amino acid substitutions were made in order to delete the motifs, using residues found at this position in human germ-line antibody sequences, unless a substitution was required within a CDR. Following this round of motif deletion, the resultant sequences were checked for generation of new MHC Class II motifs which were similarly deleted if present. The resultant de-immunised V_H and V_L sequences are shown in Figure 15. The de-immunised V_H and V_L were constructed as described for the 340 antibody by the method of Daugherty BL *et al.* (*Nucleic Acids Research* **19** 2471, 1991) using long synthetic oligonucleotides. The required sequence was synthesised as 5 or 6 long oligonucleotides (DIVH1 to DIVH6 and DIVK1 to DIVK5, shown in Figure 16) with complementary overlapping ends of 18 base pairs. These were used in PCR with two external primers (DIVH7, DIVH8, DIVK6, DIVH7, shown in Figure 16) resulting in the formation and subsequent amplification of full length V regions (351 bp for V_H and 321 bp for V_L). DNAs of the vectors M13-VHPCR1 and M13-VKPCR1 (Orlandi R, Gussow D, Jones P, Winter G. *Proc. Nat'l. Acad. Sci. USA*, **86** 3833 (1989)) were used as templates to produce a further two overlapping PCR fragments for each of V_H and V_L including 5' flanking sequence with the murine heavy chain immunoglobulin promoter and encoding the leader signal peptide (primers VHVK1 and DIVH9 for V_H, VHVK1 and DIVK8 for V_L, shown in Figure 16) and 3' flanking sequence including a splice site and intron sequences (primers DIVH10 and DIVH11 for V_H , DIVK9 and DIVK10 for V_L, shown in Figure 16). The DNA fragments so produced for each of V_H and V_L were combined in a second PCR using outer flanking primers (VHVK1 and DIVH11 for V_H, VHVK1 and DIVK10 for V_L, shown in Figure 16) to obtain the required full length DNA sequences. Cloning, sequencing, addition of human C regions and expression in NS0 cells was as for the 340 antibody (Example 1).

Example 4

A set of vaccine molecules were constructed based on the 708 antibody. As before, the various V_H and V_L molecules were assembled from long synthetic oligonucleotides using the method of PCR recombination (Daugherty *et al, ibid.*). Cloning, sequencing, addition of human IgG1 and κ constant regions and expression in NS0 cells was as for the 340 antibody (Example 1).

The first antibody vaccine ("Vaccine 1") comprised the 708 heavy and light chains from which all potential human T cell epitopes have been removed from both antibody chains, using the method described in Example 1, including epitopes found in the CDRs, apart from the region encompassing CDRs 2 and 3 and framework 3 of the heavy chain which contains the desired human epitopes. The antibody chains were not "veneered" to remove B cell epitopes. The resultant protein sequences are shown in Figure 17. The oligonucleotides for assembly of 708 Vaccine 1 V_H and V_K are shown in Figure 18. The primary PCR used oligonucleotides VHDT322F, VHDT446F, VHDT570F, VHDT340R, VHDT463R, VHDT587R, VKDT570F, VH261F and VH611R for V_H and oligonucleotides VKDT340R, VKDT322F, VKDT463R, VKDT446F, VKDT587R, VKDT570F, VK261F and VK12 resulting in the formation and subsequent amplification of full length V regions (350 bp for V_H and 396 bp for V_L). DNAs of the vectors M13-VHPCR1 and M13-VKPCR1 (Orlandi *et al., ibid.*) were used as templates to produce a further two overlapping PCR fragments for V_H including 5' flanking sequence with the murine heavy chain immunoglobulin promoter and encoding the leader signal peptide (primers VHVK1 and VH276R) and 3' flanking sequence including a splice site and intron sequences (primers VH597F and VH12) and one overlapping PCR fragment for V_L including 5' flanking sequence with the murine heavy chain immunoglobulin promoter and encoding the leader signal peptide (primers VHVK1 and VK275R), the 3' V_L sequences being included in the structural oligonucleotides. The DNA fragments so produced for each of V_H and V_L were combined in a second PCR using outer flanking primers (VHVK1 and VH12 for V_H, VHVK1 and VK12 for V_L) to obtain the required full length DNA sequences.

The second antibody vaccine ("Vaccine 2") comprised 708 heavy and light chains with epitopes from carcinoembryonic antigen (CEA) inserted into CDRH2 and CDRH3 and CDRL1 and CDRL3. The resultant sequence was checked using the method described in Example 1 for generation of new human T cell epitopes apart from those deliberately inserted. Single amino acid substitutions were made in the framework regions in order to remove any additional epitopes detected. The final protein sequences are shown in Figure 19. The oligonucleotides for assembly of 708 Vaccine 2 V_H and V_K are shown in Figure 20. The primary PCR used oligonucleotides VHDT340R, VHDT322F, VHCEA463R, VHCEA447F, VHCEA586R, VHCEA570F, VH261F and VH611R2 for V_H and VKCEA324F, VKCEA340R, VKCEA450F, VKCEA486R, VKCEA576F, VKCEA592R, VK261F and VK12 for V_L. 5' and 3' flanking sequences were added as described for the first antibody vaccine constructs.

The third antibody vaccine ("Vaccine 3") comprised 708 antibody with CEA and CD55 epitopes inserted. The heavy chain was as Vaccine 2, with an epitope from CD55 inserted from position 14 to 33 (Framework 1 into CDR1). The resultant sequence was checked using the method described in Example 1 for generation of new human T cell epitopes apart from those deliberately inserted. Single amino acid substitutions were made in the framework regions in order to remove any additional epitopes detected. The final protein sequence is shown in Figure 21. The light chain is as Vaccine 2. The oligonucleotides for assembly of 708 Vaccine 3 V_H are shown in Figure 22. The primary PCR used oligonucleotides VHCD322F, VHCD340R, VHCD463R, VHCEA447F, VHCEA570F, VHCEA586R, VH261F and VH611R2. 5' and 3' flanking sequences were added as described for the first antibody vaccine constructs.

A chimaeric 708 antibody was prepared to provide a control for comparison with the above antibody vaccine constructs. This consisted of 708 murine variable regions combined with human IgG1 and κ constant regions. The oligonucleotides for

assembly of 708 chimaeric V_H and V_K are shown in Figure 23. The primary PCR used oligonucleotides VHCH355R, VHCH337F, VHCH525R, VHCH507F, VH261F and VH611R for V_H and VKCH364R, VKCH345F, VKCH533R, VKCH518F, VK261F and VK12 for V_L. 5' and 3' flanking sequences were added as described for the first antibody vaccine constructs.

Example 5

The present invention provides a method for the redesign of a pre-existing therapeutic antibody to which a human immune response has been detected. The invention provides the method by which the therapeutic antibody may be characterised to identify epitopes relating to the observed immune response in humans. An example of this is provided in a humanised version of monoclonal antibody A33. The monoclonal antibody (mAb) A33 antigen is a transmembrane glycoprotein expressed in normal colonic and bowel epithelium and >95% of human colon cancers. The A33 antigen has been considered a useful target for colon cancer radioimmunotherapy and encouraging pre-clinical data documented (Heath J.K *et al.*, *Proc. Nat'l. Acad. Sci. USA* 94 469-474 (1997)). A humanised version of mAb A33 has been produced using the CDR grafting strategy described elsewhere (WO-A-9109967, Adair J.R. *et al.*). Clinical trials of the humanised antibody were conducted during which a HAMA response to humanised mAb A33 was reported in a number of patients. In the present example, the variable region protein sequences for the humanised A33 antibody (Figure 24) have been individually analysed by a novel process of peptide threading and by reference to a database of MHC-binding motifs. By these means, potentially immunogenic epitopes have been identified. In this example a method for the elimination and therefore de-immunisation of the potentially immunogenic epitopes is disclosed.

Potential MHC class II binding motifs in the variable region protein sequences of humanised antibody A33 were identified by the following method of peptide threading. The procedure involves computing a score for all possible candidate

binding motifs (peptides) by considering the predicted three-dimensional conformations and interactions between an MHC class II molecule and the peptide complex. The computed score indicates the predicted binding affinity for the particular peptide and MHC allele, and is used to predict peptides likely to bind, or not to bind, with the particular MHC allele.

The HLA-DRB1*0101 molecule is currently the only example of a class II MHC molecule for which the structure is available (Stern *et al.*, *Nature* 368 215-221 (1994)). This structure was used to predict peptide binding with HLA-DRB1. To predict peptide binding to other class II MHC alleles, models for particular alleles were constructed based on the known HLA-DRB1 structure. Models were constructed assuming the backbone structure of all class II MHC alleles are identical to HLA-DRB1. This assumption is supported by experimental data (Ghosh P. *et al.*, *Nature* 378 457-462 (1995)) and the high degree of homology between different MHC class II molecules. Models were built by identification of the sequence differences between the known HLA-DRB1 structure and the target allele. Side-chains in the known structure were replaced to match the target allele. The side-chain conformation near the binding groove were adjusted to give favourable steric and electrostatic arrangement whilst maintaining the largest possible binding pocket. The latter feature of the approach is significant in ensuring that modelled peptide side-chains are most readily accommodated within the binding groove, so reducing the number of candidate fragments rejected due to steric overlap with the MHC.

The structural data of HLA-DRB1*0101 was obtained from the Protein Data Bank (Bernstein F.C. *et al.*, *J. Mol. Biol.* 112 535-542 (1977)). The ten most frequent HLA-DRB1 alleles in the human Caucasian population were modelled on the HLA-DRB1*0101 structure. Homology modelling of HLA-DRB1*03011, HLA-DRB1*0302, HLA-DRB1*0401, HLA-DRB1*0801, HLA-DRB1*09011, HLA-DRB1*11011, HLA-DRB1*1201, HLA-DRB1*1301, HLA-DRB1*1401 and HLA-DRB1*15011 was conducted using molecular the modelling package "Quanta"

(Molecular Simulations Inc, University of York, England). Side-chain conformations in amino acids differing between a particular target allele and the HLA-DRB1*0101 solved structure were adjusted interactively. In most cases, torsion angles were chosen to result in minimal or nil steric overlap between mutated residues and surrounding atoms. Where non-conserved residues which were either charged, or carry side-chains able to form hydrogen bonds, were required to be inserted into the model, the potential to form favourable interactions was also considered.

All possible overlapping 13 amino-acid peptides from the humanised A33 antibody variable region protein sequences were examined. Each peptide sequence was used to form a three-dimensional model of the candidate peptide in complex with the given MHC allele. Peptide model structures were built assuming a backbone conformation and location relative to the MHC backbone structure identical to that of the previously solved structure for HLA-DRB1 in complex with an influenza haemagglutinin protein (Stern L.J. *et al.*, *ibid.*). This assumption is supported by available evidence (Jardetzky T.S. *et al.*, *Nature* **368** 711-718 (1994); Ghosh P. *et al.*, *ibid.*). Side-chains in the peptide were modelled automatically to match the sequence of the peptide under investigation, and the conformational space of each side chain was explored automatically to minimise or eliminate steric overlap and unfavourable atomic contacts, whilst also maximising favourable atomic contacts.

A score for each peptide was computed based upon the predicted inter-atomic contacts between peptide and MHC residues. Pair-wise residue-residue interaction scores were used to reward and penalise specific inter-residue contacts. The geometric constraints imposed on the peptide by the shape of the MHC binding groove play an important part of the scoring function. To reflect this, the scoring function awards favourable packing arrangements, whilst interactions involving steric overlap are penalised. Published data (Ghosh P. *et al.*, *ibid.;* Stern L.J. *et al.*, *ibid.*; Marshall K.W. *et al.*, *J. Immunol.* **152** 4946-4957 (1994); Hammer J. *et al.*, *Cell* **74** 197-203 (1993); Sinigaglia F. & Hammer J. *Current Opin. Immunol.* **6** 52-56 (1994)] indicate that larger pockets

within the MHC class II binding groove are more important in determining which peptides can bind compared with smaller pockets. The scores contributed by each pocket are also weighted based on pocket size. Peptides with the highest scores are predicted to be the best binders to the particular MHC allele.

Results from this approach are given by way of examples in Tables 1-4. These tables show output from the peptide threading process for heavy and light chains against HLA-DRB1*0101 and HLA-DRB1*03011 alleles only, although threading was performed using structural models compiled for a total of 11 HLA-DRB1 alleles. Following subtraction of sequence strings in the variable regions which are present in a database of human germline immunoglobulin variable region genes, four regions containing potential MHC class II binding motifs in the heavy and light chain humanised A33 variable regions are identified. This result is concordant with comparative searching of an MHC-binding motif database as resident on the world wide web site *wehil.wehi.edu.au*.

The potential MHC class II binding motifs identified by the use of peptide threading and corroborated with MHC-binding motif database searching were eliminated from the humanised A33 variable region protein sequences by amino-acid substitutions at specific residues (Figures 24 and 25). For the heavy chain substitution of L for I (amino acid single letter codes) at position 89, T for S at position 87, F for Y at position 91 and T for A at position 28 results in elimination of all but one of the potential epitopes. A single heavy chain epitope remains within CDRH3 as alteration may be prejudicial to the antigen binding function of A33. The method of the present invention allows for substitutions to proceed empirically. For the light chain one potential binding motif falls entirely within CDRL1, remaining potential epitopes are eliminated by substitution of F for I at position 83, S for T at position 46, G for Q at position 105 and Y for F at position 87.

Table 1

Peptides from humanised A33 light chain variable region predicted by peptide threading to have the strongest binding interaction with HLA-DRB1*0101. Shaded cells indicate peptides not present in a database of human germline immunoglobulin variable regions and hence peptides with greatest immunogenic potential in HLA-DRB1*0101 individuals.

Rank	*Sequence Position*	*Peptide Sequence*	*Score*
1	73	FTISSLQPEDIAT	2906947
2	37	QQKPGLAPKTLIY	2869068
3	56	TGVPSRFSGSGSG	2227314
4	9	SSLSVSVGDRVTI	2151680
5	76	SSLQPEDIATYFC	1981125
6	11	LSVSVGDRVTITC	1851329
7	91	HWSYPLTFGQGTK	1799889
8	96	LTFGQGTKVEVKR	1789663
9	60	SRFSGSGSGTDFT	1781975
10	45	KTLIYLASNRHTG	1665759
11	34	AWYQQKPGLAPKT	1579725
12	3	QMTQSPSSLSVSV	1548170
13	1	DIQMTQSPSSLSV	1523983
14	27	QNVRTVVAWYQQK	1479591
15	18	RVTITCKASQNVR	1404588
16	33	VAWYQQKPGLAPK	1384902
17	17	DRVTITCKASQNV	1196170
18	6	QSPSSLSVSVGDR	1134256
19	24	KASQNVRTVVAWY	1100038
20	90	QHWSYPLTFGQGT	1045861

Table 2

Peptides from humanised A33 light chain variable region predicted by peptide threading to have the strongest binding interaction with HLA-DRB1*03011. Shaded cells indicate peptides not present in a database of human germline immunoglobulin variable regions and hence peptides with greatest immunogenic potential in HLA-DRB1*03011 individuals.

Rank	*Sequence Position*	*Peptide Sequence*	*Score*
1	27	QNVRTVVAWYQQK	4679664
2	37	QQKPGLAPKTLIY	2614050
3	81	FDFATYFCQQHWS	2538553
4	45	KTLIYLASNRHTG	2214414
5	56	TGVPSRFSGSGSG	2152389
6	17	DRVTITCKASQNV	2108642
7	73	FTISSLQPEDIAT	2105806
8	60	SRFSGSGSGTDFT	2097225
9	54	RHTGVPSRFSGSG	2067916
10	96	LTFGQGTKVEVKR	2039455
11	9	SSLSVSVGDRVTI	2020864
12	8	PSSLSVSVGDRVT	1994849
13	24	KASQNVRTVVAWY	1946688
14	76	SSLQPEDIATYFC	1901925
15	11	LSVSVGDRVTITC	1812157
16	31	TVVAWYQQKPGLA	1797465
17	1	DIQMTQSPSSLSV	1638069
18	6	QSPSSLSVSVGDR	1608168
19	18	RVTITCKASQNVR	1322137
20	51	ASNRHTGVPSRFS	1291927

Table 3

Peptides from humanised A33 heavy chain variable region predicted by peptide threading to have the strongest binding interaction with HLA-DRB1*0101. Shaded cells indicate peptides not present in a database of human germline immunoglobulin variable regions and hence peptides with greatest immunogenic potential in HLA-DRB1*0101 individuals.

Rank	*Sequence Position*	*Peptide Sequence*	*Score*
1	78	TLYLQMNSLQAED	5662707
2	3	QLLESGGGLVQPG	4552719
3	35	SWVRQAPGKGLEW	3948115
4	76	KNTLYLQMNSLQA	3782821
5	16	GSLRLSCAASGFA	3367975
6	18	LRLSCAASGFAFS	3146731
7	81	LQMNSLQAEDSAI	2880801
8	71	SRDSSKNTLYLQM	2669460
9	56	SYIYPYNDSVKGRF	2543939
10	10	GLVQPGGSLRLSC	2520655
11	84	NSLQAEDSAIYYC	2412032
12	13	QPGGSLRLSCAAS	1852553
13	1	EVQLLESGGGLVQ	1831863
14	6	ESGGGLVQPGGSL	1789461
15	30	STYDMSWVRQAPG	1690753
16	34	MSWVRQAPGKGLE	1669184
17	9	GGLVQPGGSLRLS	1635030
18	46	EWVATISSGGSYT	1591661
19	100	TVVPFAYWGQGTL	1587576
20	62	DSVKGRFTISRDS	1521740

Table 4

Peptides from humanised A33 heavy chain variable region predicted by peptide threading to have the strongest binding interaction with HLA-DRB1*0311. Shaded cells indicate peptides not present in a database of human germline immunoglobulin variable regions and hence peptides with greatest immunogenic potential in HLA-DRB1*03011 individuals.

Rank	*Sequence Position*	*Peptide Sequence*	*Score*
1	35	SWVRQAPGKGLEW	4151567
2	3	QLLESGGGLVQPG	3673867
3	16	GSLRLSCAASGFA	3244475
4	18	LRLSCAASGFAFS	3110036
5	76	KNTLYLQMNSLQA	2937467
6	46	EWVATISSGGSYT	2770382
7	84	NSLQAEDSAIYYC	2282240
8	10	GLVQPGGSLRLSC	2158781
9	71	SRDSSKNTLYLQM	2151419
10	102	VPFAYWGQGTLVT	2015801
11	49	KGLEWVATISSGG	2001941
12	81	LQMNSLQAEDSAI	1971734
13	99	TLVVPFAYWGQGT	1825539
14	1	EVQLLESGGGLVQ	1824590
15	56	SYTYYLDSVKGRF	1698015
16	59	YYLDSVKGRFTIS	1684498
17	9	GGLVQPGGSLRLS	1618110
18	62	DSVKGRFRTISRDS	1601551
19	100	LVVPFAYWGQGTL	1598301
20	32	YDMSWVRQAPGKG	1593906

Example 6

In this example the method of the present invention is used to identify and eliminate potential epitopes from the murine sequence of antibody A33 (King D. J. *et al.*, *Brit. J.*

Cancer 72 1364-1372 (1995)). The humanised version of A33 was described in example 5, in the present example the starting point is the murine A33 antibody. The sequences of the V_H and V_L of the murine A33 antibody are shown in Figure 26. A de-immunised antibody was generated by analysis of these sequences. To remove B cell epitopes, the "veneering" method of Padlan (Padlan E. A., 1991, *ibid* and EP-A-0519596) was applied whereby exposed (mE or mEx) residues in the murine A33 V_H or V_L sequences were substituted by the corresponding residues in the frameworks from the human germ-line sequences DP-3 for V_H (Tomlinson *et al.*, 1992, *ibid*) with human J_H1 and LFVK431 (Cox *et al.*,1994, *ibid*) for V_L with human $J_\kappa4$. Then, the resultant sequences were analysed by searching a database of human MHC Class II binding peptides ("motif" at the World-Wide Web site *wehil.wehi.edu.ac*) for motifs present in the veneered V_H and V_L sequences. In parallel, databases of human V_H and V_L germ-line sequences (Tomlinson *et al.*, *ibid*; Cox *et al. ibid*; other germ-line sequences retrieved from EMBL, GenBank and Swiss Protein databases) were also searched for human MHC Class II binding motifs. Motifs appearing in the veneered antibody sequence which were also present in the germ-line were not considered further. For motifs present in the veneered V_H and V_L sequences and not present in the germ-line database, single amino acid substitutions were made in order to delete the motifs, using residues found at this position in human germ-line antibody sequences, unless a substitution was required within a CDR. Following this round of motif deletion, the resultant sequences were checked for generation of new MHC Class II motifs which were similarly deleted if present. The resultant de-immunised V_H and V_L sequences are shown in Figure 27. The de-immunised V_H and V_L sequences were constructed as described for the 340 antibody (Example 1) using long synthetic oligonucleotides. Cloning, sequencing, addition of human C regions and expression in NS0 cells was as for the 340 antibody (Example 1).

Example 7

The present invention details a process whereby potentially immunogenic epitopes within a non-autologous protein may be identified and offers methodology whereby

such epitopes may be eliminated. There are a number of proven therapeutic proteins for which their therapeutic use is curtailed on account of their immunogenicity in man. In the present example the therapeutic protein streptokinase is analysed for the presence of potential MHC binding motifs and a method disclosed for the removal of a number of these from the molecule.

Streptokinase (SK) is a single chain protein of approximate molecular weight 47kDa that is produced by certain strains of β-haemolytic streptococci (Huang T.T. *et al.*, *Mol. Biol.* 2 197-205 (1989)). The protein has no inherent enzymatic activity but has considerable clinical importance owing to its ability to efficiently bind human plasminogen, potentiating its activation to plasmin and thereby promoting the dissolution of fibrin filaments in blood clots. Several studies have shown that SK is an effective thrombolytic agent in the treatment of coronary thrombosis, improving survival (ISIS-2 Collaborative Group, *Lancet* 2 349-360 (1988)) and preserving left ventricular function following myocardial infarction [ISAM Study Group, *N. Engl. J. Med.* 314 1465-1471 (1986); Kennedy J. W. *et al.*, *Circulation* 77 345-352 (1988)). Despite the undoubted therapeutic value of SK, the non-autologous origin of the protein is disadvantageous due to its immunogenicity in humans. The production of neutralising antibodies in the patient in generally limits the protein to a single use.

The following method was used to identify potential MHC class II binding motifs in streptokinase. The sequence of streptokinase was identified from the GenBank database. The sequence with accession number S46536 was used throughout (Figure 28). The sequence was analysed for the presence of potential MHC class II binding motifs by computer aided comparison to a database of MHC-binding motifs as resident on world wide web site *wehil.wehi.edu.au.*

Results of the "searching" process indicate the presence of 395 potential MHC class II binding motifs. Of these, 283 matched sequences identified in a database of human germline immunoglobulin variable region protein sequences. These epitopes were not considered further on the basis that immune responses in general are not mounted to

autologous circulating proteins such as immunoglobulins. This implies immunological tolerance to the potential T-cell epitopes present in the structure of the immunoglobulins (and indeed the majority of human proteins). Epitopes presented by non-autologous proteins such as SK which are identical or similar to motifs present in immunoglobulin proteins are likely also to be tolerated and in practice may be retained through the de-immunisation process.

Following subtraction of the human immunoglobulin protein germline motifs, the remaining 112 potential epitopes were analysed individually for similarity to non-immunoglobulin protein sequences. In practice, predicted anchor residues for each potential epitope was used in a consensus sequence search of human expressed proteins. The SwissProt and GenBank translated sequence databases were interrogated using commercially available software (DNAstar Madison, WI, USA). Epitopes identified in known circulating human proteins were not considered further and were therefore allowed to remain unchanged within the SK molecule. An example of one such rejected potential epitope is given by the sequence LLKAIQEQL at positions 79-87 in the SK protein. This sequence represents a predicted consensus binding motif for HLA-DR1*0101 with anchor residues underlined. Database searching using the consensus sequence LxxxAxxxxL identifies >4000 entries in a human protein sub-set of the SwissProt database, including serum albumin protein (SwissProt accession number P02768). An example of an epitope where no match to a human protein considered to be in the general circulation was found is provided by sequence YVDVNTN at position 299-305 in the SK protein. This sequence represents a potential epitope for presentation by HLA-DR4*0401. Consensus sequence searching identifies <50 human proteins containing this motif, of which many are intracellular proteins of differentiated tissues such as brain. These may be considered as not generally available to the immune system to gain tolerance and therefore identify this as a potential epitope for elimination according to the method of the present invention. Similarly, a further potential HLA-DR1*0101 binding motif was identified in the SK peptide sequence KADLLKAI at positions 76-83 of the SK

protein. This motif identifies <150 human proteins in the same data set and was also identified for modification by the method of the present invention.

The net result of these processes was to identify those residues within the SK molecule which should be altered to eliminate potential MHC class II binding motifs. Individual amino acids within the predicted binding motifs were selected for alteration. With the object of maximising the likelihood of maintaining protein functional activity, in all cases conservative amino acid substitutions were chosen at any given site. A new (de-immunised) SK sequence was compiled (Figure 29) and further analysed by database comparison, as previously, for confirmation of successful elimination of potential MHC class II binding motifs.

The following method was used for the construction of de-immunised SK molecules. PCR primers SK1 (5'-ggaattcatgattgctggacctgagtggctg) and SK2 (5'-tggatccttatttgtcgttagggtatc) were used to amplify the wild-type SK gene from a strain of *Streptococcus equisimililis* group C (ATCC accession number 9542). The resulting 1233bp fragment was cloned into pUC19 as a *Bam*HI-*Eco*RI restriction fragment using standard techniques (Sambrook J., Fritisch E. F. & Maniatis T. (eds) in: "Molecular Cloning: A Laboratory Manual", Cold Spring Harbor Laboratory Press, NY, USA (1989)]. The gene sequence was confirmed to be identical to database entries using commercially available reagent systems and instructions provided by the supplier (Amersham, Little Chalfont, UK). Site directed mutagenesis was conducted using synthetic oligonucleotides and the "quick-change" procedure and reagents from Stratagene UK Ltd. Mutated (de-immunised) versions of the gene were confirmed by sequencing. Mutated SK genes were sub-cloned as *Eco*RI-*Bam*HI fragments into the bacterial expression vector pEKG-3 (Estrada M. P. *et al.*, *Bio/Technology* 10 1138-1142 (1992)) for expression of de-immunised SK. Recombinant protein was purified using a plasminogen affinity column according to the method of Rodriguez *et al.*, [Rodriguez P. *et al.*, *Biotechniques* 7 638-641 (1992)). Fibrinolytic activity was assessed using the casein/plasminogen plate technique and the *in vitro* clot lysis assay as described by Estrada *et al.*, (Estrada *et al.*, *ibid.*).

CLAIMS

1. A method of rendering a protein, or part of a protein, non-immunogenic, or less immunogenic, to a given species, the method comprising:

(a) determining at least part of the amino acid sequence of the protein;

(b) identifying in the amino acid sequence one or more potential epitopes for T-cells ("T-cell epitopes") of the given species; and

(c) modifying the amino acid sequence to eliminate at least one of the T-cell epitopes identified in step (b) thereby to eliminate or reduce the immunogenicity of the protein when exposed to the immune system of the given species.

2. A method as claimed in claim 1, which is a method of rendering an immunoglobulin non- or less immunogenic.

3. A method as claimed in claim 1, which is a method of rendering a variable (V) region of an immunoglobulin non- or less immunogenic.

4. A method as claimed in claim 1 or 3, which is a method of rendering a constant (C) region of an immunoglobulin non- or less immunogenic.

5. A method as claimed in any one of claims 1 to 4, which additionally comprises compromising potential epitopes for B-cells of the given species.

6. A method as claimed in claim 1, which is a method of rendering a therapeutic protein other than an immunoglobulin non- or less immunogenic.

7. A method as claimed in claim 6, wherein the therapeutic protein is a thrombolytic agent.

8. A method as claimed in claim 7, wherein the thrombolytic agent is streptokinase.

9. A method as claimed in any one of claims 1 to 8, which is a method of reducing the immunogenicity of a protein of a first species in relation to the immune system of a second species.

10. A method as claimed in claim 9, wherein the first species is a rodent species.

11. A method as claimed in claim 9, wherein the first species is non-mammalian.

12. A method as claimed in claim 9, wherein the first species is bacterial.

13. A method as claimed in any one of claims 1 to 12, wherein the given or second species is human.

14. A method as claimed in claim 3, wherein the V region is present in a whole Ig light (κ or λ) or heavy (γ, α, μ, δ or ε) chain, a light/heavy chain dimer, an SCA (single-chain antibody), an antibody or an antibody fragment.

15. A method as claimed in claim 14, wherein the antibody fragment is a Fab, $F(ab')_2$, Fab', Fd or Fv fragment.

16. A method as claimed in claim 1, comprising:

(a) determining the amino acid sequence of the V region of a non-human antibody;

(b) optionally modifying the amino acid sequence to change those non-CDR residues on the exposed surface of the antibody structure to the corresponding human amino acids taken from a reference human V region sequence;

(c) analysing the amino acid sequence to identify potential T-cell epitopes and modifying the amino acid sequence to change one or more residues in order to eliminate T-cell epitopes including those within CDRs if this does not undesirably reduce or eliminate binding affinity or undesirably alter specificity; and

(d) optionally adding human C regions to create a complete antibody which is substantially non-immunogenic.

17. A method as claimed in any one of claims 1 to 16, which includes a sequence comparison with a database of MHC-binding motifs.

18. A method as claimed in any one of claims 1 to 17, wherein the identification of T-cell epitopes comprises a computational threading method.

19. A method as claimed in any one of claims 1 to 18, wherein the amino acid sequence is modified by recombinant DNA techniques.

20. A method as claimed in any one of claims 1 to 19, wherein T-cell epitope elimination is achieved by conversion of one or more amino acids to germ-line amino acids of the given or second species at positions corresponding to those within the identified T-cell epitope(s).

21. A method as claimed in any one of claims 1 to 20, wherein the amino acid as modified or as proposed to be modified sequence is reanalysed for T-cell epitopes and optionally further modified to eliminate any newly created T-cell epitopes.

22. A method as claimed in claim 2 or 3, wherein the variable region amino acid sequence other than the CDRs comprises fewer than 70 amino acid residues identical to an acceptor human variable region sequence, in the sense used in EP-B-0451216.

23. A method as claimed in claim 2, 3 or 22, wherein the variable region amino acid sequence other than CDRs excludes amino acids from the starting antibody which are rare at the corresponding position in human immunoglobulins or which are adjacent to CDRs or which have a side-chain capable of interacting with the antigen or with the CDRs of the de-immunised antibody.

24. A method of analysing a pre-existing protein to predict the basis for immunogenic responses thereto, the method comprising identifying in the amino acid sequence of the protein one or more potential epitopes for T-cells, and optionally one or more potential epitopes for B-cells, of a given species, and inducing B- or T-cell tolerance thereto.

25. A method as claimed in claim 24, wherein the protein is an antibody or other specific binding molecule.

26. A molecule resulting from a method as claimed in any one of claims 1 to 25.

27. A molecule of a first species, at least part of which molecule is modified to the minimum extent necessary to eliminate epitopes for T-cells, and optionally also epitopes for B-cells, of a second species.

28. A molecule as claimed in claim 27, which comprises at least a variable region of an immunoglobulin of the first species, and wherein the variable region is so modified.

29. A molecule as claimed in claim 25 or 28 comprising one or more constant (C) regions from the given or second species.

30. A molecule as claimed in claim 29 for use in medicine or diagnosis.

31. The use of a molecule as claimed in any one of claims 26 to 29 in the manufacture of an therapeutic or diagnostic agent.

32. The use as claimed in claim 31, wherein the agent is an antibody or other specific binding molecule.

33. The use of a molecule as claimed in any one of claims 26 to 29 in *in vivo* or *in vitro* diagnosis.

FIGURE 1

DNA sequences of 340 V_H and V_L

340 V_H

GAAGTGCAGCTGGTGGAGTCTGGGGGAGGCTTAGTGAAGGCTGGAGGGTCCCTGAAA
CTCTCCTGTGCAGCCTCTGGATTCGCTTTCGATACCTATGACATGTCTTGGGTTCGC
CAGACTCCGGAGAAGAGGCTGGAGTGGGTCGCATACATTGGTAGTGGTGGTGATAGA
ACCTACTATCCAGACACTGTGAAGGGCCGATTCACCATTTCCAGAGACAATGGCAAG
AACACCCTGTATTTGCAATTGAACAGTCTGAAGTCTGAGGACACAGCCATGTATTAC
TGTGCAAGACATTATGGTCACTACGTGGACTATGCTGTGGACTACTGGGGTCAAGGA
ACCTCAGTCACCGTCTCCTCA

340 V_L

ACATTGTGCTGACACAGTCTCCTGCTTCCTTAGCTGTATCTCTGGGGCAGAGGGCCA
CCATCTCATACAGGGCCAGCAAAAGTGTCAGTACATCTGGCTATAGTTATATGCACT
GGAACCAACAGAAACCAGGACAGCCACCCAGACTCCTCATCTATCTTGTATCCAACC
TAGAATCTGGGGTCCCTGCCAGGTTCAGTGGCAGTGGGTCTGGGACAGAGTTCACCC
TCAACATCCATCCTGTGGAGGAGGAGGATGCTGCAACCTATTACTGTCAGCACATTA
GGGAGCTTATCACGTTCGGAGGGGGGACCAAGCTGGAAATAAAA

28. A molecule as claimed in claim 27, which comprises at least a variable region of an immunoglobulin of the first species, and wherein the variable region is so modified.

29. A molecule as claimed in claim 25 or 28 comprising one or more constant (C) regions from the given or second species.

30. A molecule as claimed in claim 29 for use in medicine or diagnosis.

31. The use of a molecule as claimed in any one of claims 26 to 29 in the manufacture of an therapeutic or diagnostic agent.

32. The use as claimed in claim 31, wherein the agent is an antibody or other specific binding molecule.

33. The use of a molecule as claimed in any one of claims 26 to 29 in *in vivo* or *in vitro* diagnosis.

FIGURE 1

DNA sequences of 340 V_H and V_L

340 V_H

GAAGTGCAGCTGGTGGAGTCTGGGGGAGGCTTAGTGAAGGCTGGAGGGTCCCTGAAA
CTCTCCTGTGCAGCCTCTGGATTCGCTTTCGATACCTATGACATGTCTTGGGTTCGC
CAGACTCCGGAGAAGAGGCTGGAGTGGGTCGCATACATTGGTAGTGGTGGTGATAGA
ACCTACTATCCAGACACTGTGAAGGGCCGATTCACCATTTCCAGAGACAATGGCAAG
AACACCCTGTATTTGCAATTGAACAGTCTGAAGTCTGAGGACACAGCCATGTATTAC
TGTGCAAGACATTATGGTCACTACGTGGACTATGCTGTGGACTACTGGGGTCAAGGA
ACCTCAGTCACCGTCTCCTCA

340 V_L

ACATTGTGCTGACACAGTCTCCTGCTTCCTTAGCTGTATCTCTGGGGCAGAGGGCCA
CCATCTCATACAGGGCCAGCAAAAGTGTCAGTACATCTGGCTATAGTTATATGCACT
GGAACCAACAGAAACCAGGACAGCCACCCAGACTCCTCATCTATCTTGTATCCAACC
TAGAATCTGGGGTCCCTGCCAGGTTCAGTGGCAGTGGGTCTGGGACAGAGTTCACCC
TCAACATCCATCCTGTGGAGGAGGAGGATGCTGCAACCTATTACTGTCAGCACATTA
GGGAGCTTATCACGTTCGGAGGGGGGACCAAGCTGGAAATAAAA

FIGURE 2

Protein sequence of 340 murine VH and VL.

```
Murine 340 VH  EVQLVESGGGLVKAGGSLKLSCAASGFAFDTYDMSWVRQTPEKRLEWVAYI
Murine 340 VL  DIVLTQSPASLAVSLGQRATISYRASKSVSTSGYSYMHWNQQKPGQPPRLL

Murine 340 VH  GSGGDRTYYPDTVKGRFTISRDNGKNTLYLQLNSLKSEDTAMYYCARHYGH
Murine 340 VL  IYLVSNLESGVPARFSGSGSGTEFTLNIHPVEEEDAATYYCQHIRELITFG

Murine 340 VH  YVDYAVDYWGQGTSVTVSS
Murine 340 VL  GGTKLEIK
```

FIGURE 3

Protein sequence of humanised 340 VH and VL

```
Humanised 340 VH    EVQLVESGGGLVQPGGSLRLSCAASGFAFDTYDMSWVRQAPGKGLEWVAYI
Humanised 340 VL    EIVLTQSPATLSLSPGERATLSYRASKSVSTSGYSYMHWNQQKPGQAPRLL

Humanised 340 VH    GSGGDRTYYPDTVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARHYGH
Humanised 340 VL    IYLVSNLESGIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQHIRELITFG

Humanised 340 VH    YVDYAVDYWGQGTTVTVSS
Humanised 340 VL    GGTKVEIK
```

FIGURE 4

Oligonucleotides for construction of humanised 340 VH and VK

Long Oligonucleotides:

VH1

5'GACATGTCATAGGTATCGAAAGCGAATCCAGAGGCTGCACAGGAGAGTCTCAGGG
ACCCTCCAGGCTGCACTAAGCCTCCCCCAGACTCCACCAGCTGCACTTC3'

VH2

5'CGATACCTATGACATGTCTTGGGTTCGCCAGGCTCCGGGGAAGGGGCTGGAGTGG
GTCGCATACATTGGTAGTGGTGGTGATAGAACCTACTATCCAGACACTG3'

VH3

5'GGCTGTGTCCTCAGCCCTCAGACTGTTCATTTGCAAATACAGGGAGTTCTTGGCA
TTGTCTCTGGAAATGGTGAATCGGCCCTTCACAGTGTCTGGATAGTAGG3'

VH4

5'AGGGCTGAGGACACAGCCGTGTATTACTGTGCAAGACATTATGGTCACTACGTGG
ACTATGCTGTGGACTACTGGGGTCAAGGAACCACAGTCACCGTCTCCTCA3'

VK1

5'AGATGTACTGACACTTTTGCTGGCCCTGTATGAGAGGGTGGCCCTCTCCCCCGGA
GATAGAGATAAGGTAGCAGGAGACTGTGTCAGCACAATCTC3'

VK2

5'GCAAAAGTGTCAGTACATCTGGCTATAGTTATATGCACTGGAACCAACAGAAACC
AGGACAGGCACCCAGACTCCTCATCTATCTTGTATCCAACCTA3'

VK3

5'CGGCTCCAGAGAAGAGATGGTGAGGGTGAAGTCTGTCCCAGACCCACTGCCACTG
AACCTGGCAGGGATCCCAGATTCTAGGTTGGATACAAGATA3'

VK4

5'ATCTCTTCTCTGGAGCCGGAGGATTTTGCAGTCTATTACTGTCAGCACATTAGGG
AGCTTATCACGTTCGGAGGGGGGACCAAGGTGGAAATAAAA3'

Short Flanking Primers:-

VH5

5'GAAGTGCAGCTGGTGGAGTC3'

VH6

5'TGAGGAGACGGTGACTGTGG3'

VK5

5'GAGATTGTGCTGACACAGTC3'

VK6

5'TTTTATTTCCACCTTGGTCC3'

For 5' flanking sequence from VHPCR1 and VKPCR1:

VH/VK1

5'AAGCTTATGAATATGCAAAT3'

VH7

5'CACCAGCTGCACTTCGGAGTGGACACCTGTG3'

VK7

5'TGTCAGCACAATCTCGGAGTGGACACCTGTG3'

FIGURE 4 (Cont'd)

For 3' flanking sequence from VHPCR1 and VKPCR1:

VH8

5'GTCACCGTCTCCTCAGGTGAGTCCTTACAA3'

VH9

5'GCGGATCCTATAAATCTCTG3'

VK8

5'AAGGTGGAAATAAAACGTGAG3'

VK9

5'GCGGATCCAACTGAGGAAGC3'

FIGURE 5

Protein sequence of de-immunised 340 VH and VL

340 VH EVQLVESGGGLVQPGGSLRLSCAASGFTFDTYDMSWVRQAPGKGLEWVAYI
340 VK EIVLTQSPATLAVSPGEKATISYRASKSVSTSGYSYMHWNQQKPGQPPRLL

340 VH GSGGDRTYYPDTVKGRFTISRDNAKNTLYLQMNSLRAEDTAVYYCARHYGH
340 VK IYLVSNLESGVPARFSGSGSGTDFTLTISSVEPEDAATYYCQHIRELITFG

340 VH YVDYAVDYWGQGTTVTVSS
340 VK GGTKLEIK

FIGURE 6

Oligonucleotides for construction of de-immunised 340 VH and VK

Long Oligonucleotides:

VH1

5'GACATGTCATAGGTATCGAAAGTGAATCCAGAGGCTGCACAGGAGAGTCTCAGGG
ACCCTCCAGGCTGCACTAAGCCTCCCCCAGACTCCACCAGCTGCACTTC3'

VH2

5'CGATACCTATGACATGTCTTGGGTTCGCCAGGCTCCGGGGAAGGGGCTGGAGTGG
GTCGCATACATTGGTAGTGGTGGTGATAGAACCTACTATCCAGACACTG3'

VH3

5'GGCTGTGTCCTCAGCCCTCAGACTGTTCATTTGCAAATACAGGGTGTTCTTGGCA
TTGTCTCTGGAAATGGTGAATCGGCCCTTCACAGTGTCTGGATAGTAGG3'

VH4

5'AGGGCTGAGGACACAGCCGTGTATTACTGTGCAAGACATTATGGTCACTACGTGG
ACTATGCTGTGGACTACTGGGGTCAAGGAACCACAGTCACCGTCTCCTCA3'

VK1

5'AGATGTACTGACACTTTTGCTGGCCCTGTATGAGATGGTGGCCTTCTCCCCCGGA
GATACAGCTAAGGTAGCAGGAGACTGTGTCAGCACAATCTC3'

VK2

5'GCAAAAGTGTCAGTACATCTGGCTATAGTTATATGCACTGGAACCAACAGAAACC
AGGACAGCCACCCAGACTCCTCATCTATCTTGTATCCAACCTA3'

VK3

5'CGGCTCCACAGAACTGATGGTGAGGGTGAAGTCTGTCCCAGACCCACTGCCACTG
AACCTGGCAGGGACCCCAGATTCTAGGTTGGATACAAGATA3'

VK4

5'ATCAGTTCTGTGGAGCCGGAGGATGCTGCAACCTATTACTGTCAGCACATTAGGG
AGCTTATCACGTTCGGAGGGGGGACCAAGCTGGAAATAAAA3'

Other primers are as for the humanised genes apart from:

VK6

5'TTTTATTTCCAGCTTGGTCC3'

VK8

5'AAGCTGGAAATAAAACGTGAG3'

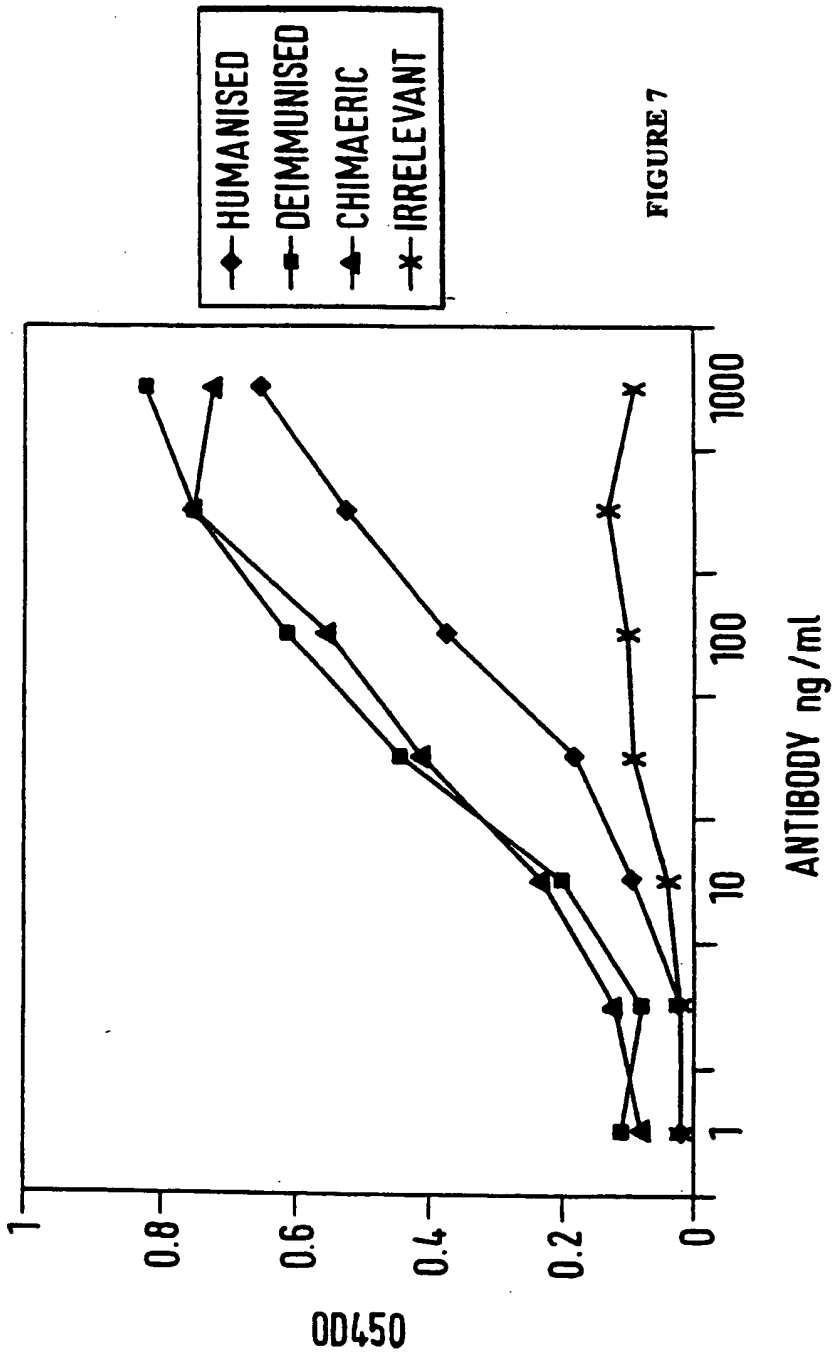
HUMANISED
DEIMMUNISED
CHIMAERIC
IRRELEVANT
OD450
0
0.2
0.4
0.6
0.8
1
1
10
100
1000
ANTIBODY ng/ml

FIGURE 7

FIGURE 8

Protein sequence of humanised 340 VH compared with the sequence with murine epitopes inserted

```
HUMANISED 340 VH EVQLVESGGGLVQPGGSLRLSCAASGFAFDTYDMSWVRQAPGKGL
ALTERED 340 VH   EVQLVESGGGLVQPGGSLRLSCAASGFAFDTYDMSWVRQAPGKGL

HUMANISED 340 VH EWVAYIGSGGDRTYYPDTVKGRFTISRDNAKNSLYLQMNSLRAED
ALTERED 340 VH   EWVAYIGSGGDRTYYPDTVKGRFTISRDNAKNSLSLQMNSLRAED

HUMANISED 340 VH TAVYYCARHYGHYVDYAVDYWGQGTTVTVSS
ALTERED 340 VH   TAVYYCARHYGHYVDYAVDYWGQGSTVTVSS
```

FIGURE 9

Oligonucleotide primers for insertion of murine epitopes into humanised 340 VH by SOE PCR

Mutagenic Oligonucleotides:

5'TCCCTGTCTTTGCAAATGAA3'

5'CATTTGCAAAGACAGGGAGTTCT3'

5'TCAAGGATCCACAGTCACCG3'

5'ACTGTGGATCCTTGACCCCA3'

Flanking Oligonucleotides:

5'AAGCTTATGAATATGCAAAT3'

5'GCGGATCCTATAAATCTCTG3'

FIGURE 10

Protein sequence of mouse deimmunised 340 VH

EVQLVESGGGLVKAGGSLKLSCAASGFAFDTYDMSWVRQTPEKRLEWVAYIGSGGDR
TYYPDTVKGRFTISRDNGKNSLYLQMNSLKSEDTAMYYCARHYGHYVDYAVDYWGQG
TSVTVSS

FIGURE 11

Oligonucleotide primers for construction of mouse deimmunised VH

VH1
5'GACATGTCATAGGTATCGAAAGCGAATCCAGAGGCTGCACAGGAGAGTTTCAGGGA
CCCTCCAGCCTTCACTAAGCCTCCCCCAGACTCCACCAGCTGCACTTC3'

VH2
5'CGATACCTATGACATGTCTTGGGTTCGCCAGACTCCGGAGAAGAGGCTGGAGTGGG
TCGCATACATTGGTAGTGGTGGTGATAGAACCTACTATCCAGACACTG3'

VH3
5'GGCTGTGTCCTCAGACTTCAGACTGTTCATTTGCAAATACAGGGAGTTCTTGCCAT
TGTCTCTGGAAATGGTGAATCGGCCCTTCACAGTGTCTGGATAGTAGG3'

VH4
5'AAGTCTGAGGACACAGCCATGTATTACTGTGCAAGACATTATGGTCACTACGTGGA
CTATGCTGTGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCA3'

VH5
5'GAAGTGCAGCTGGTGGAGTC3'
VH6
5'TGAGGAGACGGTGACTGAGG3'

Other primers are as for Humanised VH

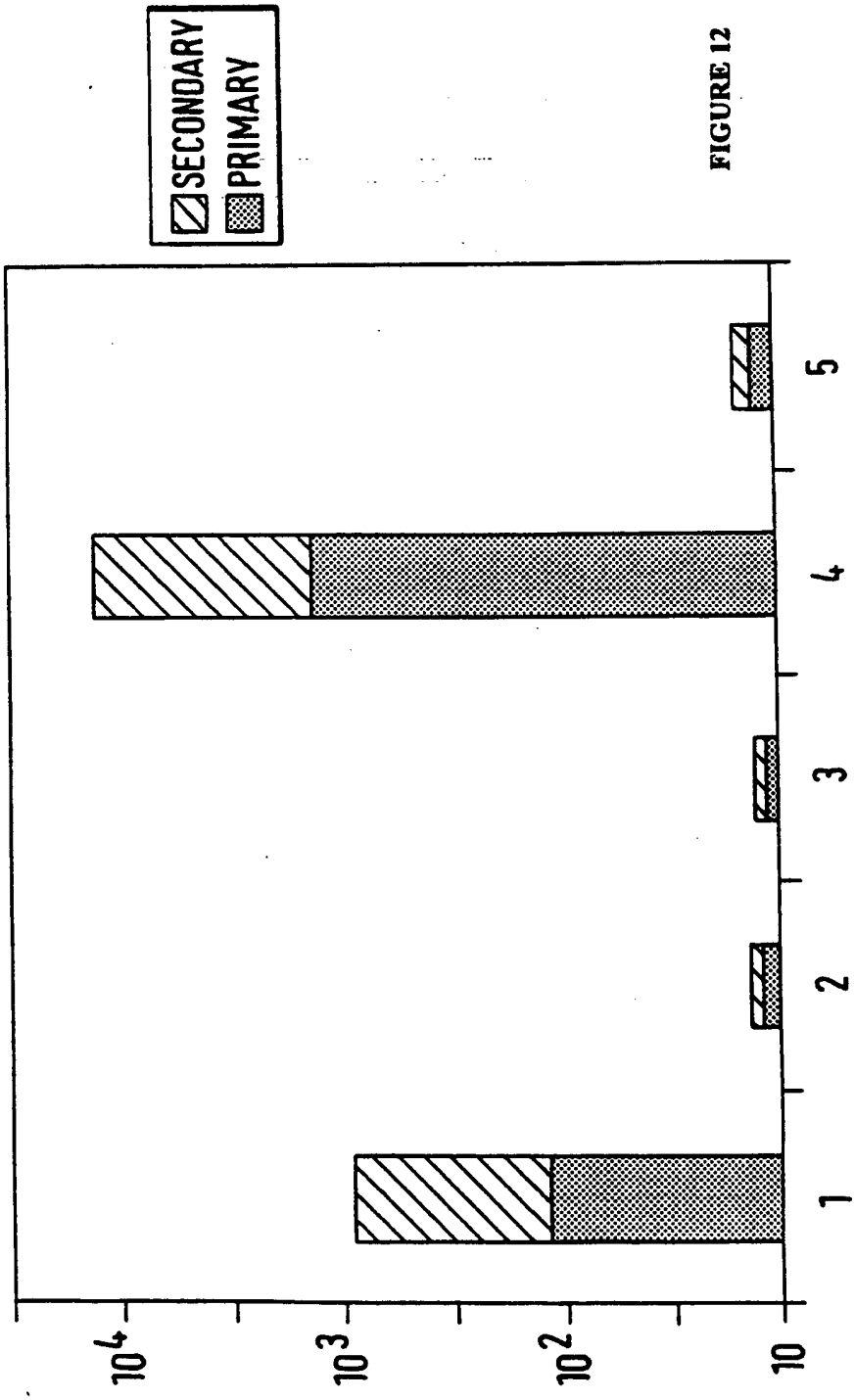

FIGURE 12

FIGURE 13

DNA sequences of murine 708 V_H and V_L

708 V_H

GAGGTCCAGCTGCAACAGTCTGGACCTGAGCTGGTGAAGCCTGGGGCTTCAGTGAAG
ATATCCTGTAAGACTTCTGGACACACATTCACTGAATACAACATGCAGTGGGTGAAG
CAGAGCCTTGGACAGAGCCTTGAGTGGATTGGAGGTATTAATCCTAACAATGTTGGT
TCTATCTACAACCAGAAGTTCAGGGGCAAGGCCACATTGACTGTAGACAAGTCCTCC
AGCACAGCCTACATGGAGCTCCGCAGCCTGACATCTGAGGATTCTGCAGTCTATTAC
TGTGCAAGAGGCTATGGTAACTACGTGGCTTACTGGGGCCAAGGGACTCTGGTCACT
GTCTCTGCA

708 V_L

GACATTGTGATGACCCAGTCTCAAAAATTCATGTCCACATCAGTAGGAGACAGGGTC
AGCGTCACCTGCAAGGCCAGTCAGAATGTGAATACTAATGTAGCCTGGTATCAACAG
AAACCAGGGCAATCTCCTAAATCACTGATTTACTCGGCATCCTACCGATACAGTGGA
GTCCCTGATCGCTTCACAGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGC
AATGTGCAGTCTGAAGACTTGGCAGAGTTTTTCTGTCAGCAATATAACAGGTATCCG
TTCACGTTCGGTGGTGGGACCAAGCTGGAGCTGAAA

FIGURE 14

Protein sequence of murine 708 V_H and V_L.

Murine 708 VH

EVQLQQSGPELVKPGASVKISCKTSGHTFTEYNMQWVKQSLGQSLEWIGGINPNNVG
SIYNQKFRGKATLTVDKSSSTAYMELRSLTSEDSAVYYCARGYGNYVAYWGQGTLVT
VSA

Murine 708 VL

DIVMTQSQKFMSTSVGDRVSVTCKASQNVNTNVAWYQQKPGQSPKSLIYSASYRYSG
VPDRFTGSGSGTDFTLTISNVQSEDLAEFFCQQYNRYPFTFGGGTKLELK

FIGURE 15

Protein sequence of de-immunised 708 V_H and V_L

De-immunised 708 V_H

EVQLVQSGPGLVQPGGSVRISCATSGHTFSEYNMQWVKQAQGKGLEWMGGINPNNVG
SIYNQKFRGRFTLSVEKSKNTAYMQLSSLKSEDSAVYYCARGYGNYVAYWGQGTLVT
VSS

De-immunised 708 V_L

DIQMTQSPSSMSTSVGDRVTVTCKASQNVNTNVAWYQQKPGKSPQSLIYSASYRYSG
VPSRFSGSGSGTDFTLTISSVQPEDFAEYYCQQYNRYPFTFGGGTKLELK

FIGURE 16

Oligonucleotides for construction of De-immunised 708 V_H and V_L

Long Oligonucleotides:

DIVH1

5'TGTCCAGAAGTCGCACAGGATATCCTCACTGAACCCCCAGGCTGCACCAGCCCAG
GTCCAGACTGTACCAGCTGGACCTC3'

DIVH2

5'CTGTGCGACTTCTGGACACACATTCTCTGAATACAACATGCAGTGGGTGAAGCAG
GCCCAAGGAAAGGGCCTTGAGTGG3'

DIVH3

5'AACCTGCCCCTGAACTTCTGGTTGTAGATAGAACCAACATTGTTAGGATTAATAC
CTCCCATCCACTCAAGGCCCTTTCC3'

DIVH4

5'GAAGTTCAGGGGCAGGTTCACATTGTCTGTAGAGAAGTCCAAGAACACAGCCTAC
ATGCAGCTCAGCAGCCTGAAATCTG3'

DIVH5

5'TTGGCCCCAGTAAGCCACGTAGTTACCATAGCCTCTTGCACAGTAATAGACTGCA
GAATCCTCAGATTTCAGGCTGCTGA3'

DIVH6

5'GTGGCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTTCA3'

DIVK1

5'TGACTGGCCTTGCAGGTGACGGTGACCCTGTCTCCTACTGATGTGGACATGGAGC
TTGGAGACTGGGTCATCTGAATGTC3'

DIVK2

5'CACCTGCAAGGCCAGTCAGAATGTGAATACTAATGTAGCCTGGTATCAACAGAAA
CCAGGGAAATCTCCTCAATCACTGA3'

DIVK3

5'CCCAGATCCACTGCCTGAGAAGCGACTAGGGACTCCACTGTATCGGTAGGATGCC
GAGTAAATCAGTGATTGAGGAGATT3'

DIVK4

5'TCAGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCTCTGTGCAGCCTG
AAGACTTCGCAGAGTATTACTGTCA3'

DIVK5

5'TTTCAGCTCCAGCTTGGTCCCACCACCGAACGTGAACGGATACCTGTTATATTGC
TGACAGTAATACTCTGCG3'

Short Flanking Primers:-

DIVH7

5'GAGGTCCAGCTGGTACAG3'

DIVH8

5'TGAAGAGACAGTGACCAG3'

DIVK6

5'GACATTCAGATGACCCAG3'

DIVK7

5'TTTCAGCTCCAGCTTGGT3'

FIGURE 16 (Cont'd)

For 5' flanking sequence from VHPCR1 and VKPCR1:
VH/VK1
5'GCAAGCTTATGAATATGCAAAT3'
DIVH9
5'TACCAGCTGGACCTCGGAGTGGACACCTGT3'
DIVK8
5'GGTCATCTGAATGTCGGAGTGGACACCTGT3'

For 3' flanking sequence from VHPCR1 and VKPCR1:
DIVH10
5'GTCACTGTCTCTTCAGGTGAGTCCTTACAA3'
DIVH11
5'GCGGATCCTATAAATCTCTG3'
DIVK9
5'AAGCTGGAGCTGAAACGTGAGTAGAATTTA3'
DIVK10
5'GCGGATCCAACTGAGGAAGC3'

FIGURE 17

Protein sequence of Vaccine 1 708 V_H and V_L.

708 V1 V_H

EVQLQQSGPELVKPGASVKISCKTSGYTFTEYNMNWVRQSPGQSLEWIGGINPNNVG
SIYNQKFRGKATLTVDKSSSTAYMELRSLTSEDSAVYYCARGYGNYVAYWGQGTLVT
VSA

708 V1 V_L

DIVMTQSQKFVSTSVGDRVSITCKASQNVNTNVAWYQQKPGQSPQSLIYSASYRFSG
VPDRFSGSGSGTDFTLTISNVQSEDFAEYYCQQYNSYPRTFGGGTKLELK

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FIGURE 18

Oligonucleotides for construction of Vaccine 1 708 V_H and V_L

Long Oligonucleotides:

VHDT340R

5'TATCCAGAAGTCTTACAGGATATCTTCACTGAAGCCCCAGGCTTCACCAGCTCAG
GTCCAGACTGTTGCAGCTGGACCTC3'

VHDT322F

5'CCTGTAAGACTTCTGGATACACATTCACTGAATACAACATGAACTGGGTGAGGCA
GAGCCCCGGACAGAGCCTTGAGTGG3'

VHDT446F

5'GAAGTTCAGGGGCAAGGCCACATTGACTGTAGACAAGTCCTCCAGCACAGCCTAC
ATGGAGCTCCGCAGCCTGACATCTG3'

VHDT463R

5'GCCTTGCCCCTGAACTTCTGGTTGTAGATAGAACCAACATTGTTAGGATTAATAC
CTCCAATCCACTCAAGGCTCTGTCC3'

VHDT570F

5'GTGGCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCA

VHDT587R

5'TTGGCCCCAGTAAGCCACGTAGTTACCATAGCCTCTTGCACAGTAATAGACTGCA
GAATCCTCAGATGTCAGGCTGCGGA

VKDT322F

5'TAACCTGCAAGGCCAGTCAGAATGTGAATACTAATGTAGCCTGGTATCAACAGAA
ACCAGGGCAATCTCCTCAATCACTG3'

VKDT340R

5'TGACTGGCCTTGCAGGTTATGCTGACCCTGTCTCCTACTGATGTGGACACGAATT
TTTGAGACTGGGTCATCACAATGTC3'

VKDT446F

5'CTCAGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCAATGTGCAGTCT
GAAGACTTTGCAGAGTATTACTGTC3'

VKDT463R

5'CCAGATCCACTGCCTGAGAAGCGATCAGGGACTCCACTGAATCGGTAGGATGCCG
AGTAAATCAGTGATTGAGGAGATTG3'

VKDT570F

5'CTGGAGCTGAAACGTGAGTAGAATTTAAACTTTGCTTCCTCAGTTGGATCCGC3'

VKDT587R

5'CTCACGTTTCAGCTCCAGCTTGGTCCCACCACCGAACGTGCGCGGATAGCTGTTA
TATTGCTGACAGTAATACTCTGCAA3'

Short Flanking Primers:-

VH261F

5'GAGGTCCAGCTGCAACAGTC3'

VH611R

5'TGCAGAGACAGTGACCAGA3'

VK261F

5'GACATTGTGATGACCCAGT3'

VK12

5'GCGGATCCAACTGAGGAAGCA3'

FIGURE 18 (Cont'd)

For 5' flanking sequence from VHPCR1 and VKPCR1:
VH/VK1
5'GCAAGCTTATGAATATGCAAAT3'
VH276R
5'GTTGCAGCTGGACCTCGGAGTGGACACCTGTG3'
VK275R
5'GGTCATCACAATGTCGGAGTGGACACCTGT3'

For 3' flanking sequence from VHPCR1:
VH597F
5'GTCACTGTCTCTGCAGGTGAGTCCTTACAAC3'
VH12
5'GCGGATCCTATAAATCTCTG3'

FIGURE 19

Protein sequence of Vaccine 2 708 V_H and V_L.

708 V2 V_H

EVQLQQSGPELVKPGASVKISCKTSGYTFTEYNMNWVRQSPGQSLEWNGGRNNSIVK
SITVSASGTKATLTVDKSSSTAYMELRSATSEDSAGIYISPSYTYYRPGVGQGTLGT
VSA

708 V2 V_L

DIVMTQSQKFVSTSVGDSASVTCTLLSVTRNDVSRYQQSPGQWPQSLIYSASYRFSG
VPDRFSGSGSGTDFTLTISNVQSEDLAEFMCYLSGANLNLTGGGTKLEVR

FIGURE 20

Oligonucleotides for construction of Vaccine 2 708 V_H and V_L

Long Oligonucleotides:

VHDT340R

5'TATCCAGAAGTCTTACAGGATATCTTCACTGAAGCCCCAGGCTTCACCAGCTCAG
GTCCAGACTGTTGCAGCTGGACCTC3'

VHDT322F

5'CCTGTAAGACTTCTGGATACACATTCACTGAATACAACATGAACTGGGTGAGGCA
GAGCCCCGGACAGAGCCTTGAGTGG3'

VHCEA447F

5'GCCTCCGGCACCAAGGCCACATTGACTGTAGACAAGTCCTCCAGCACAGCCTACA
TGGAGCTCCGCAGCGCCACATCTGA3'

VHCEA463R

5'GCCTTGGTGCCGGAGGCGGACACGGTGATAGACTTAACGATGGAGTTATTGCGAC
CTCCGTTCCACTCAAGGCTCTGTCC3'

VHCEA570F

5'CGCCCCGGCGTGGGCCAAGGGACTCTGGGCACTGTCTCTGCA3'

VHCEA586R

5'TGGCCCACGCCGGGGCGGTAGTAGGTATAGGAGGGGGAGATGTAGATGCCTGCAG
AATCCTCAGATGTGGCGCTGCG3'

VKCEA324F

5'ACCTGCACCCTGCTGTCCGTGACCCGCAACGACGTATCCCGCTATCAACAGTCCC
CAGGGCAATGGCCTCAATCACTGAT3'

VKCEA340R

5'GACAGCAGGGTGCAGGTCACGCTGGCGGAGTCTCCTACTGATGTGGACACGAATT
TTTGAGACTGGGTCATCACAATGTC3'

VKCEA450F

5'GGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCAATGTGCAGTCTGAAG
ACCTGGCAGAGTTCATGTGTTACCT3'

VKCEA486R

5'GTCCCAGATCCACTGCCTGAGAAGCGATCAGGGACTCCACTGAATCGGTAGGATG
CCGAGTAAATCAGTGATTGAGGCCA3'

VKCEA576F

5'GTGCGACGTGAGTAGAATTTAAACTTTGCTTCCTCAGTTGGATCCGC3'

VKCEA592R

5'TTCTACTCACGTCGCACCTCCAGCTTGGTCCCACCACCGGTCAGGTTCAGGTTGG
CGCCGGACAGGTAACACATGAACTC3'

Short Flanking Primers:-

VH261F

5'GAGGTCCAGCTGCAACAGTC3'

VH611R2

5'TGCAGAGACAGTGCCCAG3'

VK261F

5'GACATTGTGATGACCCAGT3'

VK12

5'GCGGATCCAACTGAGGAAGCA3'

FIGURE 20 (Cont'd)

For 5' flanking sequence from VHPCR1:
VH/VK1
5'GCAAGCTTATGAATATGCAAAT3'
VH276R
5'GTTGCAGCTGGACCTCGGAGTGGACACCTGTG3'
VK275R
5'GGTCATCACAATGTCGGAGTGGACACCTGT3'

For 3' flanking sequence from VHPCR1 and VKPCR1:
VH597F
5'GTCACTGTCTCTGCAGGTGAGTCCTTACAAC3'
VH12
5'GCGGATCCTATAAATCTCTG3'

FIGURE 21

Protein sequence of Vaccine 3 708 V_H

708 V3 V_H
EVQLQQSGPELAKFGATISFSCNTGYKLFGSTSMNRLRQSPGQSLEWNGGRNNSIVK
SITVSASGTKATLTVDKSSSTAYMELRSATSEDSAGIYISPSYTYYRPGVGQGTLGT
VSA

FIGURE 22

Oligonucleotides for construction of Vaccine 3 708 V_H

Long Oligonucleotides:
VHCD340R
5'TTGTAGCCGGTGTTGCAGGAGAAGGAGATGGTGGCGCCGAACTTCGCCAGCTCGG
GGCCGGACTGCTGCAGCTGCACCTC3'
VHCD322F
5'CTGCAACACCGGCTACAAGCTGTTCGGCTCCACCTCCATGAACCGACTTCGCCAG
TCCCCCGGCCAGTCCCTGGAGTGGA3'
VHCD463R
5'GCCTTGGTGCCGGAGGCGGACACGGTGATAGACTTAACGATGGAGTTATTGCGAC
CTCCGTTCCACTCCAGGGACTGGCC3'
VHCEA447F
5'GCCTCCGGCACCAAGGCCACATTGACTGTAGACAAGTCCTCCAGCACAGCCTACA
TGGAGCTCCGCAGCGCCACATCTGA3'
VHCEA570F
5'CGCCCCGGCGTGGGCCAAGGGACTCTGGGCACTGTCTCTGCA3'
VHCEA586R
5'TGGCCCACGCCGGGGCGGTAGTAGGTATAGGAGGGGGAGATGTAGATGCCTGCAG
AATCCTCAGATGTGGCGCTGCG3'

Short Flanking Primers:-
VH261F
5'GAGGTCCAGCTGCAACAGTC3'
VH611R2
5'TGCAGAGACAGTGCCCAG3'

For 5' flanking sequence from VHPCR1:
VH/VK1
5'GCAAGCTTATGAATATGCAAAT3'
VH276R
5'GTTGCAGCTGGACCTCGGAGTGGACACCTGTG3'

For 3' flanking sequence from VHPCR1:
VH597F
5'GTCACTGTCTCTGCAGGTGAGTCCTTACAAC3'
VH12
5'GCGGATCCTATAAATCTCTG3'

FIGURE 23

Oligonucleotides for construction of Chimaeric 708 V_H and V_L

Long Oligonucleotides:

VHCH355R

5' TATTCAGTGAATGTGTGTCCAGAAGTCTTACAGGATATCTTCACTGAAGCCCCAGGCTTC
ACCAGCTCAGGTCCAGACTGTTGCAGCTGGACCTC3'

VHCH337F

5' GACACACATTCACTGAATACAACATGCAGTGGGTGAAGCAGAGCCTTGGACAGAGCCTTG
AGTGGATTGGAGGTATTAATCCTAACAATGTTGGTTCTATCTAC3'

VHCH525R

5' CAGATGTCAGGCTGCGGAGCTCCATGTAGGCTGTGCTGGAGGACTTGTCTACAGTCAATG
TGGCCTTGCCCCTGAACTTCTGGTTGTAGATAGAACCAACATT3'

VHCH507F

5' CTCCGCAGCCTGACATCTGAGGATTCTGCAGTCTATTACTGTGCAAGAGGCTATGGTAAC
TACGTGGCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCA3'

VKCH345F

5' GTGAATACTAATGTAGCCTGGTATCAACAGAAACCAGGGCAATCTCCTAAATCACTGATT
TACTCGGCATCCTACCGATACAGTGGAGTCCCTGATCGCTTCAC3'

VKCH364R

5' CAGGCTACATTAGTATTCACATTCTGACTGGCCTTGCAGGTGACGCTGACCCTGTCTCCT
ACTGATGTGGACATGAATTTTTGAGACTGGGTCATCACAATGTC3'

VKCH518F

5' TTTCTGTCAGCAATATAACAGGTATCCGTTCACGTTCGGTGGTGGGACCAAGCTGGAGCT
GAAACGTGAGTAGAATTTAAACTTTGCTTCCTCAGTTGGATCCGC3'

VKCH533R

5' ATATTGCTGACAGAAAAACTCTGCCAAGTCTTCAGACTGCACATTGCTGATGGTGAGAGT
GAAATCTGTCCCAGATCCACTGCCTGTGAAGCGATCAGGGACTC3'

Short Flanking Primers:-

VH261F

5' GAGGTCCAGCTGCAACAGTC3'

VH611R

5' TGCAGAGACAGTGACCAGA3'

VK261F

5' GACATTGTGATGACCCAGT3'

VK12

5' GCGGATCCAACTGAGGAAGCA3'

For 5' flanking sequence from VHPCR1 and VKPCR1:

VH/VK1

5' GCAAGCTTATGAATATGCAAAT3'

VH276R

5' GTTGCAGCTGGACCTCGGAGTGGACACCTGTG3'

VK275R

5' GGTCATCACAATGTCGGAGTGGACACCTGT3'

For 3' flanking sequence from VHPCR1:

VH597F

5' GTCACTGTCTCTGCAGGTGAGTCCTTACAAC3'

VH12

5' GCGGATCCTATAAATCTCTG3'

FIGURE 24

Protein sequence of humanised A33 V_H and V_L

Humanised A33 V_H
EVQLLESGGGLVQPGGSLRLSCAASGFAFSTYDMSWVRQAPGKGLEWVATIS
SGGSYTYYLDSVKGRFTISRDSSKNTLYLQMNSLQAEDSAIYYCAPTTVVPFA
YWGQGTLVTVSS

Humanised A33 V_L
DIQMTQSPSSLSVSVGDRVTITCKASQNVRTVVAWYQQKPGLAPKTLIYLASN
RHTGVPSRFSGSGSGTDFTFTISSLQPEDIATYFCQQHWSYPLTFGQGTKVEVK

FIGURE 25

Protein sequence of de-immunised humanised A33 V_H and V_L

De-Immunised Humanised A33 V_H
EVQLLESGGGLVQPGGSLRLSCAASGFTFSTYDMSWVRQAPGKGLEWVATIS
SGGSYTYYLDSVKGRFTISRDSSKNTLYLQMNSLQAEDTALYFCAPTTVVPFA
YWGQGTLVTVSS

De-Immunised Humanised A33 V_L
DIQMTQSPSSLSVSVGDRVTITCKASQNVRTVVAWYQQKPGLAPKSLIYLASN
RHTGVPSRFSGSGSGTDFTFTISSLQPEDFATYYCQQHWSYPLTFGGGTKVEV
K

FIGURE 26

Protein sequence of murine A33 V_H and V_L

Murine A33 V_H
EVKLVESGGGLVKPGGSLKLSCAASGFAFSTYDMSWVRQTPEKRLEWVATIS
SGGSYTYYLDSVKGRFTISRDSARNTLYLQMSSLRSEDTALYYCAPTTVVPFA
YWGQGTLVTVSA

Murine A33 V_L
DIVMTQSQKFMSTSVGDRVSITCKASQNVRTVVAWYQQKPGQSPKTLIYLAS
NRHTGVPDRFTGSGSGTDFTLTISNVQSEDLADYFCLQHWSYPLTFGSGTKLE
VK

FIGURE 27

Protein sequence of de-immunised murine A33 V_H and V_L

De-immunised murine A33 V_H
EVQLVESGGEVKKPGATLKLSCKASGFTFTTYDMSWVRQAPGKGLEWVATI
SSGGSYTYYLDSVKGRFTITRDSSTNTLYLEMSSLRSEDTALYFCAPTTVVPFA
YWGQGTLVTVSS

De-immunised murine A33 V_L
DIQMTQSPSSMSTSVGDRVSITCKASQNVRTVVAWYQQKPGKSPKSLIYLAS
NRHTGVPSRFSGSGSGTDFTLTISSVQPEDFADYFCLQHWSYPLTFGGGTKLE
VK

FIGURE 28

Protein sequence of streptokinase from *Streptococcus equisimilis*

IAGPEWLLDRPSVNNSQLVVSVAGTVEGTNQDISLKFFEIDLTSRPAHGGKTEQGLSPKS
KPFATDSGAMPHKLEKADLLKAIQEQLIANVHSNDDYFEVIDFASDATITDRNGKVYFAD
KDGSVTLPTQPVQEFLLSGHVRVRPYKEKPIQNQAKSVDVEYTVQFTPLNPDDDFRPGLK
DTKLLKTLAIGDTITSQELLAQAQSILNKTHPGYTIYERDSSIVTHDNDIFRTILPMDQE
FTYHVKNREQAYEINKKSGLNEEINNTDLISEKYYVLKKGEKPYDPFDRSHLKLFTIKYV
DVNTNELLKSEQLLTASERNLDFRDLYDPRDKAKLLYNNLDAFGIMDYTLTGKVEDNHDD
TNRIITVYMGKRPEGENASYHLAYDKDRYTEEEREVYSYLRYTGTPIPDNPNDK

FIGURE 29

Protein sequence of a de-immunised streptokinase molecule

IAGPEWLLDRPSVNNSQLVVSVAGTVEGTNQDISLKFFEIDLTSRPAHGGKTEQGLSPKS
KPFATDSGAMPHKLEKADLLKAKQEQLIANVHSNDDYFEVIDFASDATITDRNGKVYFAD
KDGSVTLPTQPVQEFLLSGHVRVRPYKEKPIQNQAKSVDVEYTVQFTPLNPDDDFRPGLK
DTKLLKTLAIGDTITSQELLAQAQSILNKTHPGYTIYERDSSIVTHDNDIFRTILPMDQE
FTYHVKNREQAYEINKKSGLNEEINNTDLISEKYYVLKKGEKPYDPFDRSHLKLFTIKFV
DVNTNELLKSEQLLTASERNLDFRDLYDPRDKAKLLYNNLDAFGIMDYTLTGKVEDNHDD
TNRIITVYMGKRPEGENASYHLAYDKDRYTEEEREVYSYLRYTGTPIPDNPNDK

INTERNATIONAL SEARCH REPORT

Inter ɔnal Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K16/46 C07K14/315 G01N33/563 A61K39/395

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K G01N A61K

Documentation searched other than minimumdocumentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of theinternational search	Date of mailing of the international search report
24 September 1998	01/10/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cervigni, S

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INTERNATIONAL SEARCH REPORT

.national application No.
PCT/GB 98/01473

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 33 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1 [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

.formation on patent family members

Inter onal Application No
PCT/GB 98/01473

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Form PCT/ISA/210 (patent family annex) (July 1992)